Viability of *Giardia intestinalis* cysts:
assessing viability under environmental conditions

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Abstract

Much work has been put into the detection and monitoring of *Giardia*, but once found, it is not easy to tell whether the cysts are viable and thus infective. There are fluorescently labelled monoclonal antibody kits which can be used to identify *Giardia*, but are the *Giardia* cysts viable?

Excystation has been the main method used to determine the viability of cysts. This is quite unreliable as varying excystation conditions seem to be required for different strains of cysts. Using samples of fresh cysts, certain batches consistently measured 80-95% viable, while others resulted in viability measurements of 0-10%. The cysts themselves displayed the normal morphology of viable cysts. The assumption that partially excysted trophozoites as well as completely excysted trophozoites are viable may also lead to over-estimation of viable cyst numbers.

Another commonly used method for estimating the viability of *Giardia* is staining with vital dyes, in particular the combination of fluorescein diacetate (FDA) and propidium iodide (PI). These also gave unexpected results where none of the cysts in a fresh sample stained with FDA, which usually stains viable cysts. An alternative dye, 4',6-diamidino-2-phenylindole (DAPI) was used in the place of FDA. The combination of DAPI and PI showed viabilities of 85.7% for cyst samples. This correlated well with 88% viability using excystation.

Using the DAPI/PI combination, the viability of *G. intestinalis* cysts over time was monitored under different temperature conditions, and in sea water. Temperature was quite significant in the viability of the cysts – cysts stored at 4°C remained viable for 62 days, while those stored at 25°C were non-viable after 5 days. Sea-water had an immediately lethal effect on the *G. intestinalis* cysts, with all cysts non-viable after 45 minutes.

Giardia intestinalis trophozoites can be cultured in the laboratory. By the addition of bile to the growth media, it is possible to transform these into cysts. Over the course of four days in encystation media, a large proportion of the trophozoites in the culture were

converted into cysts, 3.5 X 10^5 cysts/ml from an initial trophozoite concentration of 7.2 X 10^5 organisms/ml. However, the cysts generated from the strains of *G. intestinalis* used were completely non-viable, compared with viability rates for fresh *in vivo* cysts of 80-95%.

A population of hamsters was found to be carrying a *Giardia* which seemed different to recognised species. An analysis was carried out by PCR and sequencing of sections of the ribosomal DNA of this *Giardia*. Through this it was found to be closely related to *Giardia muris*, but perhaps not as closely related as to be a species of *G. muris*, possibly a sub-species. The rDNA analysis used may be very useful in typing other strains and species of *Giardia*.

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Chapter 1. Introduction

1.1 The *Giardia* organism

Giardia is the name given to the organism which causes the gastrointestinal disease giardiasis. Giardiasis is endemic in many areas of the world, with prevalence rates of 2-5% in industrialised countries and perhaps 20-30% in developing countries (Farthing, 1994). It has been reported as the most commonly occurring waterborne disease in the U.S.A. and is also widespread in New Zealand waterways. Since 1996, giardiasis has also become a notifiable disease in New Zealand.

Giardia causes intestinal infection in mammals, birds, reptiles and amphibians (Adam, 1991). The organism was first described by Antony van Leeuwenhoek in 1681 when he examined his own diarrheal stool:

> "My excrement being so thin, I was . . . persuaded to examine it . . . wherein I have sometimes also seen animalcules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood-globule, but all of the one and the same make; their bodies were somewhat longer than broad and their belly, which was flatlike, furbished with sundry paws, wherewith they made such a stir in the clear medium, and among the globules, that you might e'en fancy you saw a pissabed running up against a wall; and albeit they made a quick motion with their paws, yet for all that they made but small progress." (Dobell, 1920)

Lambl made a more detailed description of the *Giardia* trophozoite in 1859, and the *Giardia* species which infects humans was named after him (Adam, 1991). *Giardia lamblia* is also known as *Giardia intestinalis* and *Giardia duodenalis*. In this thesis, the species of *Giardia* which affects humans will be referred to as *Giardia intestinalis*.

The *Giardia* organism is found in two forms. The trophozoite form is found in the gut of an infected body. It is a binucleate organism, unilaterally symmetrical, pear-shaped, with a pair of median bodies and four pairs of flagellae (anterior, caudal, posterior and ventral). A distinctive feature of *Giardia* is the large concave ventral disk for attachment to the host's internal mucosa. This disc covers approximately two-thirds of the anterior surface of the trophozoite (Adam, 1991). It is surrounded by a flexible rim which meets the surfaces of intestinal microvilli, disrupting and deforming them (Erlandsen and Chase, 1974). This extension is known as the ventrolateral flange. Internal plates support this flange, perhaps enabling it to contract and thus give it flexibility (Thompson *et al.*, 1993). *Giardia* trophozoites have two nuclei which are placed symmetrically on either side of the middle. Both are transcriptionally active and seem to replicate at almost the same time (Adam, 1991).

The cyst organism is the infective form of *Giardia*, found outside a host body. It is highly resistant to environmental factors, and does not replicate. It is elliptical in shape, with dimensions approximately 5μm by 8μm and a cyst wall about 0.3μm thick (Adam, 1991). By scanning electron microscope, the surface of the cyst wall appears smooth. Although the cyst wall is tightly applied to the organism, little of the internal structures of viable cysts are visible under phase contrast microscopy (Feely *et al.*, 1984).

1.2 Species differentiation

Giardia is classed in the Phylum Sarcomastigophora, Class Zoomastigophora and in the Order Diplomonadida, Family Hexamitidae (Adam, 1991; Feely *et al.*, 1984). The shape of the median bodies, body size and form of the trophozoites, were used in a system devised by Filice in 1952 as a basis for distinction between different species of *Giardia*. *Giardia* had been differentiated as species by host specificity, but in many cases this specificity was questionable. Using Filice's system of classification, the number of *Giardia* species dropped from more than forty, identified on the basis of host occurrence, to three based on morphology (Filice, 1952). These were *Giardia intestinalis*, found in many mammals including humans, as well as birds and reptiles, *Giardia muris*, found in rodents, and *Giardia agilis*, which infects amphibians (Adam,

1991). Trophozoites of the species *Giardia intestinalis* measure between 10 μm and 16 μm in length, and 5 μm and 9 μm in width. The median bodies take the appearance of claw-hammers lying across the trophozoite. Those of *Giardia muris* are small and round and are seen in the centre of the body. The *G. muris* trophozoite measures 10 μm in length by 7 μm in width. *Giardia agilis* is longer and narrower, 20 μm in length and 4.5 μm in width with tear-drop shaped median bodies lying lengthways along the body (Meyer, 1994). All three species though have a similar shape and have a ventral adhesive disc, so the median body morphology is the important criterion for determining the species (Erlandsen and Bemrick, 1987).

More recently, two other species have been identified using morphological characteristics observed under scanning electron microscopy (SEM). *Giardia psittaci* from budgerigars has claw-hammer shaped median bodies, but unlike *Giardia intestinalis*, it lacks a ventro-lateral flange, and so does not have a marginal groove bordering the anterior and lateral border of the adhesive disc (Erlandsen and Bemrick, 1987). *Giardia ardeae*, which is found in great blue herons, gray herons and some other wading birds such as egrets (van Keulen *et al.*, 1992), has the typical trophozoite morphology in its pyriform shape and ventral adhesive disc, but it has a single caudal flagellum, rather than the pair found in other species, and a deep notch in the ventral adhesive disc. In addition, it has a variable median body morphology. Median bodies seen varied from the round-oval *Giardia muris* type to the claw-hammer *Giardia intestinalis* type. Chromosome migration patterns were also distinctly different for *Giardia intestinalis*, *Giardia muris* and *Giardia ardeae*, despite the fact that the morphology of *G. ardeae* is similar to that of *G. muris* with its deeply notched ventral disc (Erlandsen *et al.*, 1990). Examination of morphology alone is not sufficient to determine species within the *Giardia* genus. These findings have been borne out by electrophoretic karyotyping. DNA patterns for *G. intestinalis*, *G. muris* and *G. ardeae* are all distinctly different (Campbell *et al.*, 1990).

The rRNA of *Giardia intestinalis* are smaller than those of other eukaryotes, and are also smaller than those of the eubacteria (Adam, 1991). The rDNA gene itself is 5,566 bp and is tandemly repeated in the genome (Healy *et al.*, 1990). The tandem repeat unit includes the large subunit (LSU) (23S), small subunit (SSU) (16S) and the 5.8S subunit.

The size of the rDNA repeat in *Giardia muris* is larger than for *Giardia intestinalis* at 7,668 bp. This size difference is mainly attributable to the different length of the internal transcribed spacer region (van Keulen *et al.*, 1991). The rDNA operon of *Giardia muris* is particularly different from that of *Giardia intestinalis* and *Giardia ardeae* in that the distance between the SSU rDNA and the LSU rDNA is shorter in *G. muris*; the spacer is longer than in *G. intestinalis* and is heterogeneous; and the SSU is the shortest of the three *Giardia* and has the lowest G + C content. Examination of the rDNA operon show that *G. intestinalis* and *G. ardeae* are more closely related to each other than to *G. muris* (van Keulen *et al.*, 1993).

Weiss *et al.* (1992) examined the *Giardia* rDNA sequence, using the polymerase chain reaction. They found that strains of *G. intestinalis* from a variety of sources and locations could be divided into three groups based on defined nucleotide changes within a 183 bp fragment of the 16S rDNA unit. These groupings correlated with groupings previously made based on surface antigen patterns and restriction enzyme analysis (Weiss *et al.*, 1992). Mahbubani *et al.* (1992) devised a system using PCR directed at the giardin gene and gene probes to distinguish *G. intestinalis* from *G. muris* and *G. ardeae* (Mahbubani *et al.*, 1992). The advantages of using the PCR system are that it is very sensitive (Weiss *et al.* (1992) found that the equivalent of 1 organism's DNA was a sufficient quantity to analyse), and that it is a highly specific system so that it is not as crucial to have a pure DNA sample.

In 1995, it was discovered that a colony of hamsters kept at the ESR-CDC (Environmental Science and Research – Communicable Diseases Centre) in Keneperu were infected with *Giardia*. Initial investigations of this 'strain' using standard staining techniques (McLenachan *et al.*, in preparation) indicated possible differences when compared to *Giardia muris*. It was decided to characterise this strain further by analysing the rDNA and comparing it with the rDNA of known species.

1.3 Life cycle

Giardia has a simple, asexual life-cycle. When a host ingests a cyst, stomach acid and other gastric conditions cause the cyst to undergo a process of excystation in the host's upper small intestine (Gillin *et al.*, 1987). In this process, the cyst wall is ruptured and a mass of protoplasm is released from one pole of the cyst, which differentiates into two binucleate trophozoites (Boucher and Gillin, 1990; Meyer, 1994). The exposure of the cysts to gastric acid in the host's stomach is thought to be an important trigger for excystation although the trophozoites do not emerge in the stomach as the low pH would kill them (Boucher and Gillin, 1990). The trophozoites emerge in the duodenum and colonise the epithelium of the small intestine. In rats trophozoites have been observed to adhere to the apical and lateral surfaces of intestinal villi (Erlandsen *et al.*, 1974). *Giardia* is typically non-invasive (Meyer, 1994). The trophozoites multiply by binary fission. Their rate of multiplication varies between strains, and is also dependent on host factors such as nutritional and immune status, but tends to be rapid (Thompson *et al.*, 1993).

Encystation, the mechanism where the trophozoites are transformed into cysts, occurs as trophozoites pass through the jejunum and to the posterior areas of the small intestine. The transformation takes 44 to 72 hours (Gillin *et al.*, 1987; Thompson *et al.*, 1993). Due to this lengthy period, trophozoites can be found in diarrhoeic samples where the transit time through the intestine is short, and cysts tend to be found in formed stools (Meyer, 1994). The mechanisms of encystment are not completely understood but the exposure of trophozoites to bile salts in the lower small intestine seems to be an important stimulus for encystation (Gillin *et al.*, 1987). These stimuli cause the appearance of encystation specific vesicles (ESVs) in the trophozoites. These then transport cyst wall components to the plasma membrane of the encysting trophozoite. Cyst wall components seem to be completely absent in non-encysting trophozoites (Mowatt *et al.*, 1995). Either before or after the cyst wall is formed, asexual reproduction occurs within the cyst with nuclear division resulting in a quadrinucleate cyst. Encystation begins 4-15 days after the colonisation of a host's small intestine.

Some cysts may require a maturation time of seven or so days before they become infective (Bingham and Meyer, 1979; Schaefer *et al.*, 1984).

1.4 The Disease

Studies of travellers to Leningrad, where it was common for travellers to contract waterborne giardiasis, have indicated that the incubation period averages 1-2 weeks, but can range from 1-45 days. Where volunteers were infected with *Giardia intestinalis* trophozoites by intubation, the incubation time was one week. The disease caused by *Giardia* can range from a complete lack of symptoms, to acute short-term diarrhoea to long-term illness lasting several weeks and more. Symptoms rarely last less than one week (Adam, 1991). For most untreated patients, giardiasis is self-limiting within a 2-4 week period, but in up to 25% of documented cases, illness persists for seven weeks and longer (Farthing, 1994). Giardiasis causes diarrhoea, nausea, abdominal discomfort and bloating, and often weight loss. Sometimes early in infection, fever can occur (Adam, 1991). For those with chronic diarrhoea, weight losses of 10-20% of body weight can be experienced (Farthing, 1994). In children, giardiasis can cause protein calorie malnutrition leading to retarded growth and development (Thompson *et al.*, 1993).

Infection with *Giardia* appears to cause disease through epithelial damage. This leads to increased epithelial turnover, villous shortening and disaccharidase deficiency. In rodent models infected with *Giardia muris* and *Giardia intestinalis*, villous atrophy and damage to microvilli correlated with brush border enzyme deficiencies. These returned to normal levels once the infection was cleared. Brush border injury was indicated by lower disaccharidase activity and a decreased microvillous surface area, leading to malabsorption which in turn meant reduced growth. The degree of brush border injury and the decrease in microvillous surface area, both factors affecting disaccharidase activity, seem to correlate to the parasite load. This may in part explain the symptoms of both diarrhoea and the failure to thrive (Thompson *et al.*, 1993).

Although there are many drugs available with which to treat giardiasis, most of these were originally developed to treat other infections. The first to be used was quinacrine, the antimalarial drug, in 1937. It is still often used in the United States to treat giardiasis (Jarroll, 1994). The class of drugs mostly used today are the nitroimidazoles, including metronidazole (5-nitroimidazole), which was developed to treat trichomoniasis in 1959, and tinidazole. Another drug used is furazolidone, a synthetic nitrofuran. However, studies have shown that treatment failures can be expected using these drugs. The efficacy of the four widely used drugs metronidazole, tinidazole, furazolidone and quinacrine are: furazolidone 58-95%, tinidazole 88-100%, metronidazole 46-95% and quinacrine 60-100%.

Unfortunately, these drugs cause some unpleasant side-effects. Mecaprine (quinacrine) can cause dizziness, headaches and mild gastrointestinal illness. Long-term administration of quinacrine can give rise to chronic dermatoses which can be lichenoid, eczmatoid or exfoliative. It may also cause anaemia. The nitroimidazoles, such as metronidazole and tinidazole can bring on gastrointestinal upset, headache, rashes and sometimes furred tongue, vertigo, urethral discomfort and darkening of the urine. As metronidazole can cross the placental barrier, it is not recommended during the first trimester of pregnancy. Paromomycin is recommended for pregnant women. Gastro-intestinal upsets are the only known side-effects, but this is rare. Side effects are also rare for furazolidone, but it may cause headaches, nausea and vomiting (Boreham, 1994).

1.5 Faecal-oral transmission

Giardiasis can be contracted by faecal-oral transmission and by waterborne transmission. The rate of infection with *Giardia* seems to be dependent on socio-economic status. In addition, the incidence of giardiasis is higher in urban areas rather than in rural ones. One study of giardiasis in New Zealand showed an urban incidence 2.3 times the rate of positive samples in rural areas (Walker *et al.*, 1991). This may however indicate lower rates of patients seeing doctors, and a lower likelihood of doctors requesting faecal samples in rural areas. It may also point to a more significant

role for faecal-oral transmission in urban environments where interpersonal contact is more frequent. An infective dose may be as low as 10 cysts, so that very little contaminating matter is required for transmission of *Giardia* to occur. Where the patient experiences severe diarrhoea, it is possible that disease can be transmitted through trophozoites as well as by cysts (Thompson *et al.*, 1993). A survey of New Zealand medical laboratories in 1990 indicated that there were at least 3,356 cases of *Giardia* a year (Walker *et al.*, 1991). These figures are probably gross underestimates, as a high number of cases are asymptomatic.

Factors which increase the chance of contracting giardiasis include overcrowding, high population density, poor hygiene standards, a lack of potable water and a lower level of education. *Giardia* is thus particularly prevalent in developing countries. All children in a sample group in rural Guatemala were found to have been infected by the age of three. By the age of six months, 40% of a sample group of children in Peru were found to have been infected with *Giardia.* Prevalence rates among children in Zimbabwe and Bangladesh were found to be approximately 20% (Adam, 1991). Disadvantaged groups in developed countries are also at risk, with a prevalence rate of 20-60% found among the Australian Aborigine population, with the higher rates particularly common in children (Thompson *et al.,* 1993). Children in institutionalised care, such as day-care centres, also seem to have a higher incidence of giardiasis than the general population. The risk factors in such situations are lack of toilet-training and personal hygiene (Keystone *et al.,* 1978). Prevalence of *Giardia* may be as high as 35% in some institutions (Thompson *et al.*, 1993). The children usually have asymptomatic infections but transmission to family members may result in symptomatic giardiasis (Adam, 1991). Food-borne transmission through an infected food handler is a well-recognised source of infection (Thompson *et al.*, 1993). Direct faecal-oral transmission probably also accounts for increasing rates of giardiasis in homosexual men (Adam, 1991).

1.6 Waterborne transmission

Giardia is so widespread in the environment that all surface water is subject to contamination by human or animal sources. A test carried out in fourteen U.S. states and one Canadian province found *Giardia* cysts in 69 out of 85 samples (81.2%) collected from 66 water treatment plants (LeChevallier *et al.*, 1991b). *Giardia* was first found in New Zealand among returning servicemen in the 1940's (Ampofo *et al.*, 1991). In research recently carried out in New Zealand, the country was divided into fifty-six grids and samples of fresh water taken from each grid in accordance with the USEPA method. Thirty-one of the fifty-six areas (55.4%) produced samples positive for *Giardia*. Some areas were sampled more than once; overall, 22.6% of the samples were positive for *Giardia* (Brown *et al.*, 1997). In New Zealand, there is a general lack of toilet facilities on tramping routes and in roadside rest areas resulting in people using anywhere convenient, such as behind bushes. This seems to be one way that *Giardia* is spreading into environments in which there is otherwise little human activity (Ampofo *et al.* 1991). Such sources can enable *Giardia* to spread into waterways and also expose animals to *Giardia*-infected faeces.

Although waterborne *Giardia* cysts can be inactivated by disinfectants, they are one of the most resistant waterborne pathogens. In the 90 waterborne outbreaks that occurred in the U.S. between 1965 and 1984, many of the treatment systems for the contaminated water supplies involved little more than disinfection with chlorine (Jakubowski, 1988). The effectiveness of chlorine, which is one of the most common disinfectants used for water supplies in inactivating cysts depends on the pH, temperature, water turbidity and chlorine contact time (Walker *et al.*, 1991). A variation in any one of these factors can result in treatment failure; considering the number of factors, the chance of failure is quite high. A number of communities in New Zealand, whose water sources are uncontaminated by wastewater discharge and appear pristine rely on chlorination alone or catchment closure or control for water treatment (Ampofo *et al.*, 1991). *Giardia* cysts however are well able to survive in clear, cold water. Although chlorine concentrations of 3 ppm are considered cysticidal, the standard chlorine levels in New

Zealand drinking water are 0.5 ppm (Walker *et al.*, 1991). Alone, routine levels of chlorine used in disinfection of drinking water are not adequate to inactivate *Giardia* cysts.

1.7 Zoonoses

A variety of animals, both domestic and wild, can serve as hosts for *Giardia*, and *Giardia* and *Cryptosporidium* are commonly found in farm animals in Canada (Olson *et al.*, 1997). The run-off from farms has been implicated in the contamination of waterways in the U.S.A. (LeChevallier *et al.*, 1991a). In the U.S.A. and in Canada, muskrats and beavers are thought to play a role as reservoir hosts of *Giardia*. Studies have found the prevalence of *Giardia* infection to be about 15% in beavers and up to 95% in muskrats (Jakubowski, 1988). Testing in New Zealand has shown that birds, domestic and feral animals here also carry *Giardia intestinalis*, which could be infectious to humans (Marino, 1993). A study of cats and dogs in Palmerston North and Hamilton found that 3% to 25% were infected with *Giardia* (Tonks *et al.*, 1991), while in a similar study in Perth 21% of dogs and 14% of cats were infected (Swan and Thompson, 1986).

However, although there are many reports of animals infected with *Giardia intestinalis*, the evidence on whether zoonotic transmission can occur is conflicting. Common domestic pets such as cats and dogs would appear to be prime candidates for zoonotic transmission of disease, and *Giardia* has been found in both. Yet in one study of an Aboriginal community where there was close physical association between humans and dogs, the *Giardia intestinalis* from the two populations were genetically dissimilar (Hopkins *et al.*, 1997). A single dog had a mixed infection including *Giardia intestinalis* of the type-sequence found in the human group, so it was possible that cross-transmission took place, although on a low level. On the other hand, the study by Isaac-Renton *et al.* (1993) found that human isolates of *Giardia* from an outbreak of giardiasis were genetically identical to isolates collected from diseased beavers caught at the water intake location for the affected town (Isaac-Renton *et al.*, 1993). From the

variety and number of animals carrying *Giardia*, it seems likely that other animals are also likely to be capable of transmitting *Giardia* to humans.

1.8 Culturing *Giardia*

Karapetyan carried out the first successful culturing of *Giardia in vitro* in 1960, growing *Giardia intestinalis* symbiotically with chick fibroblasts and *Candidia guilliermondii*. In 1970, Meyer achieved the isolation and axenic cultivation of *Giardia intestinalis* from rabbit, chinchilla and cat (Meyer, 1976). Early cultures were obtained from trophozoites isolated from the gut of animals. In 1979, Bingham and Meyer reported isolating trophozoites through the excystation of cysts from faeces. These were subsequently maintained for 7 months, with most cultivated axenically (Bingham and Meyer, 1979). This method made it possible to cultivate different strains of *Giardia* from a stool specimen from an infected human or animal, but due to the fastidious nature of *Giardia*, there are still relatively few successes in cultivating trophozoites. The difficulties of establishing axenic cultures is highlighted by Brown *et al.* (1992). Eight New Zealand strains of *Giardia intestinalis* were established from 129 attempts. The cultures themselves take several weeks to become well established (Brown *et al.*, 1992).

Establishing *Giardia* as a viable and axenic laboratory culture is a very time-consuming exercise, with no guarantee of success. Some strains, such as *Giardia intestinalis* from dogs, are more difficult than others. In the study by Mayrhofer *et al.*, (1992) it was found that different populations predominate in mice than in culture. In some cases, *in vitro* culturing resulted in a single genotype of *Giardia*, which on inoculation into suckling mice was discovered to be a mixture of genotypes (Mayrhofer *et al.*, 1992). Selective pressures during culturing may mean the loss of a predominant strain during the processing, giving a false idea of the make-up of the original population. Established *in vitro* cultures may lead to conclusions which are irrelevant for the majority of uncultured *Giardia*, such as susceptibility to drugs (Upcroft *et al.*, 1994).

Almost all *Giardia* cultures established *in vitro* successfully have been of the *Giardia intestinalis* species. *Giardia ardeae* was established as an axenic culture by Erlandsen *et al.* (1990), and there has been a report of axenic cultivation of *Giardia muris*, a species which has not been successfully cultivated in the past. Some strains of *Giardia intestinalis*, such as *Giardia* from dogs, are also notoriously difficult to culture. The problem of what ingredients to include in the complex medium took many years of experimentation, but Gillin and Diamond's modified TYI-S-33 medium, containing bovine serum, cysteine and bile is now the standard medium used in the cultivation of *Giardia* (Meyer *et al.*, 1987). L-cysteine, a thiol reducing compound, is highly important for attachment of the trophozoites to the surface of cell culturing containers and for survival. The presence of serum also stimulates attachment and growth of the trophozoites (Gillin and Reiner, 1982).

Different isolates of *Giardia* have different growth requirements. To start with, the initial cell concentration (ICC) needed to establish an isolate *in vitro* were found to differ for two different isolates studies by Binz *et al.*, (1992). This may mean that one isolate would require fewer cysts to establish an infection within a host *in vivo*. The two isolates also had different pH requirements and grew at different rates. The gastrointestinal pH varies widely in different parts of the intestine. While the gastric pH is quite low at 1.0-2.5, the mean pH in the proximal small intestine – the area which trophozoites tend to colonise – is 6.6 ± 0.5, the terminal ileum is pH 7.5 ± 0.4 and past the ileocecal valve is 6.4 ± 0.4. Different strains colonise different areas of the small intestine in the same host, which may be reflected in the differing growth requirements (Binz *et al.*, 1992). These are factors which may need to be considered in cultivating strains of *Giardia* which are difficult to grow and maintain in culture.

1.9 Viability

Giardia, once found, is not easily cultivated. With bacteria it is possible to identify the organism present, and also to ascertain the viable numbers by culturing on agar. The procedure is not as simple for *Giardia*. The positive identification of *Giardia* was

formerly quite a problem. There are now fluorescently labelled monoclonal antibody kits available which can do this reliably. The problem still remains of determining whether *Giardia* cysts are viable or non-viable.

For the study of viability of cysts from waterborne outbreaks, animal models have been used extensively. While mice are excellent models for the study of *Giardia muris* (Hoff *et al.*, 1985) mice cannot be infected with *Giardia intestinalis* cysts. Neo-natal mice can be infected with *Giardia intestinalis* trophozoites but this requires that cysts first be excysted *in vitro*, for which large numbers of cysts are needed. Gerbils can be infected with *Giardia intestinalis* cysts, and are very convenient for work with *Giardia*, as they can be infected with low infectious doses of cysts (ID_{50} 5-15 cysts have been reported) and the prepatent time and pathogenesis are similar to those of the original hosts (Wallis, 1994). There are some disadvantages with using animal infectivity. Not all *Giardia intestinalis* isolates have produced infection in gerbils, and some isolates while infecting the animals, have not produced cysts (Schaefer, 1988). In any case, animal infectivity is not very useful in quantitative measurements of cyst viability. Unfortunately, gerbils are not permitted in New Zealand, so alternative methods of determining viability and thus infectivity are needed.

Although excystation of *Giardia* had been observed earlier, there was no reproducible method of *in vitro* excystation prior to Bingham and Meyer's report of 1979. Many other methods of excystation have since been reported but they all follow the general pattern of attempting to duplicate *in vivo* conditions that cysts would encounter within a host organism. The conditions in a host's stomach are approximated *in vitro* by a half hour acid-induction step, followed by a reducing step, as the cysts would encounter as they passed into the small intestine.

One of the problems with excystation is that large numbers of cysts are required to ensure a statistically significant sample size, and to overcome losses in numbers during the process. Water samples seldom yield high numbers of cysts, so alternative methods are needed with which it will be possible to ascertain the viability of small numbers of cysts. One of the other methods used to determine the viability of cysts included the use of dyes such as eosin and trypan blue. A comparison of excystation with eosin

exclusion found that the numbers of cysts excluding eosin (viable cysts) was consistently higher than the number of viable cysts as counted by excystation (Bingham *et al.*, 1979).

Schupp and Erlandsen in 1987 reported on the use of the fluorogenic dyes fluorescein diacetate (FDA) and propidium iodide (PI), based on the method of Jones and Sneft (1985), to determine the viability of *Giardia muris* cysts. Comparison of the staining behaviour of the cysts with mouse infectivity and excystation showed that cysts which stained with PI (non-viable cysts) were incapable of infecting mice, and were not observed to excyst. Cysts which stained with FDA (viable cysts) were seen to excyst, releasing FDA-staining trophozoites (Schupp and Erlandsen, 1987a). Schupp and Erlandsen (1987b) also found a direct correlation between FDA/PI staining behaviour and the morphology of the cysts as viewed under differential interference contrast (DIC) and brightfield optics.

Sauch *et al.* (1991) demonstrated good correlation between cysts which stained with PI and a lack of excystation in cysts which had been exposed to heat or to a quaternary ammonium compound. However they found that there was no correlation between PI staining and lack of excystation for cysts which had been exposed to chlorine and monochloramines (Sauch *et al.*, 1991). This was also the finding of Donaghy (1993), who in addition found no correlation between excystation and fluorogenic dye staining in cysts exposed to sea water. After 1-2 days the readings using FDA indicated 100% viability, while the PI readings indicated 0% viability (Donaghy, 1993). Smith and Smith (1989) found that FDA over-estimated cyst viability, while PI under-estimated non-viable cysts compared with *in vitro* excystation. One isolate in their study did not stain at all with either PI or FDA, but underwent excystation (Smith and Smith, 1989).

The difficulties in measuring viability mean there have been few studies done on the length of time and the conditions under which *Giardia* cysts can remain viable. There has been a report of *Giardia muris* cysts stored for a year in a faecal slurry being capable of causing *Giardia* infection in rats, but other studies have not come near reproducing this. Bingham and Meyer (1979) used excystation to determine viability. Cysts remained viable for 77 days in water at 8°C (Bingham and Meyer, 1979). In DeRegnier

et al.'s (1989) study, *Giardia* cysts suspended in either lake or river water in the winter at temperatures below 10°C remained viable for 56 to 84 days and that cysts exposed to tap water were non-viable by the 14th day. Measurement of water quality parameters showed that the only factor of significance in the viability of cysts in environmental water such as in lakes and rivers was the water temperature. Low water temperatures prolonged the survival of the cysts. The viability readings using the fluorogenic dyes fluorescein diacetate and propidium iodide correlated reasonably well with animal infectivity tests (DeRegnier *et al.*, 1989). This thesis seeks to find a reproducible method of measuring cyst viability using fluorogenic dyes.

Chapter 2. Materials and Methods

2.1 Recovery of *Giardia* cysts from faecal samples

Materials:

0.01% Tween 20 solution in reverse osmosis (RO) water.

1M sucrose solution (342.3 g/L)

RO water

Meridian Merifluor *Giardia* monoclonal antibody kit

Method:

A small amount of faecal matter, 1-2 grams, was mixed with 20 ml of 0.01% Tween 20 in a 50 ml centrifuge tube. This was vortexed to break up the clumps and the solution made up to 50 ml. This was centrifuged at 1000 g for 5 minutes. The supernatant was decanted and made up to 50 ml with Tween 20 solution, vortexed, centrifuged and decanted again. This time the solution was made up to 20ml with Tween 20 solution and vortexed. Using a 5 ml pipettor, the faecal solution was underlaid with 15ml of 1M sucrose solution using a pipettor, taking care not to disturb the interface. The solution was centrifuged for 10 minutes at 500 g without brake. Aliquots of 5 ml of liquid were pipetted from the interface, 5 ml from below the interface and 5 ml from above the interface. These fractions were combined in a fresh 50 ml centrifuge tube. This step was carried out immediately after centrifuging as the sucrose is detrimental to the viability of the cysts. The fractions were made up to 50 ml with RO water and centrifuged at 1000 g for 5 minutes. The supernatant was removed. This washing step was repeated twice. The pellet was resuspended in 2 ml of RO water, and the cysts enumerated under a phase contrast microscope at 400X magnification using a haemocytometer. The cysts were stored in RO water at 4°C.

For a fluorescent monoclonal antibody stain of a sample extracted from faeces, 50 µL of the extract was pipetted onto a cavity slide and placed into a drying cupboard until dry. From the Merifluor monoclonal antibody kit, a one in ten dilution was made of the

primary reagent, 5 µL of reagent and 45 µL phosphate buffered saline (PBS), on parafilm. The 50 µL of dilute primary reagent was pipetted on to the prepared slide and left for 30 minutes in a dark chamber. The solution was rinsed off with PBS in a coplin jar, and the slide left to air-dry. A one in ten dilution of the secondary reagent was made as for the primary reagent, and 50 µL placed onto the slide. This was left for 30 minutes in the dark chamber, then rinsed with PBS. Once airdried, a drop of DPX mounting solution was added and a coverslip placed on top. This was then examined under a fluorescent microscope using a blue filter with an excitation wavelength of 450-490 nm.

2.2 Maintaining *Giardia intestinalis* cultures

2.2.1 Cultivation of *G. intestinalis* trophozoites

Materials:

G. intestinalis culture in cell-culture tubes

Filter sterilisation unit

TY1-S-33 Medium:

Trypticase Soy Broth	20.0 g
Yeast extract	10.0 g
Glucose	10.0 g
NaCl	2.0 g
K_2HPO_4	1.0 g
KH_2PO_4	0.6 g
L-Cysteine Monohydrochloride (Sigma)	1.5 g
Ferric Ammonium Citrate	0.023 g
L-Ascorbic acid	0.2 g
NCTC 135	0.94 g
Bile Bacteriological	0.8 g
Benzyl penicillin	0.06 g
Gentamycin sulphate	0.05 g
Vancomycin	0.02 g
Bovine serum	100 ml
Distilled water to	1 L

Method

To make the TY1-S-33 medium, the dry ingredients were made up in a small volume of water. The serum was added and then distilled water to make up the desired volume. This was stirred with a magnetic stirrer and the pH adjusted to between 6.8 and 7.0. The media was sterilised by positive pressure membrane filtration. Initially, it was filtered through a number of non-sterile membranes, firstly 7 layers of Whatman filter paper, then through a 0.45 μm pore size nitrocellulose filter, then a 0.2 μm pore size nitrocellulose filter. It was sterilised by filtration through a sterile 0.2 μm pore size Supor membrane. The filter unit, with the membrane in place, was sterilised by autoclaving at 121°C for 15 minutes. The media was stored at 4°C and used for up to 2 weeks.

The serum was extracted from bovine blood obtained from the freezing works. To extract the serum, the blood was left to clot overnight in cheesecloth bags at 4°C which were suspended over buckets. The bags containing the blood were then squeezed into the buckets. The blood was centrifuged at 1000 g for 15 minutes and left to stop without the brake. The serum layer was drawn off the top and stored at -20°C.

Where trophozoites were cultured from excysted cysts, the antibiotic Amphotericin B was used to minimise fungal growth. This was added to the TY1-S-33 medium to a final concentration of 10 μg/ml.

The trophozoites were grown in 70 ml cell culture tubes at 37°C. Every three days, the media in the cell culture tubes was decanted and the tubes refilled with fresh TY1-S-33 pre-warmed to 37°C. About once a month, the cultures were transferred to fresh cell culture containers. Using a trophozoite culture in the late log phase of growth, about three days old, the media was decanted off and the tube half-filled with fresh cold TY1-S-33 media at 4°C and left for ten minutes. This was examined under an inverted microscope to ensure that the trophozoite monolayer had lifted off the tube surface. The medium was then poured into a fresh culture tube and topped up with fresh TY1-S-33 media held at 37°C. If more than one tube was required, the cold media containing trophozoites was divided between two fresh culture tubes, which were then topped up

with fresh media. The fresh tubes were incubated at 37°C. To grow trophozoites in bulk, 600 ml cell culture tubes were used.

2.2.2 Cryopreservation of *G. intestinalis* trophozoites

Materials:

15% Dimethyl sulphoxide (DMSO) in TY1-S-33 medium

Sterile 250 ml centrifuge tubes

TYI-S-33 medium

1 ml cryopreservation tubes (Nunc)

Method:

Trophozoites in the log phase of growth, about 3 days old, were used. The supernatant of the tubes was poured off into sterile centrifuge tubes. The growth tubes were then half-filled with cold TY1-S-33 growth media held at 4°C and left for five to ten minutes until most of the trophozoites had detached. This was then also poured off into the sterile centrifuge tubes. The trophozoites were concentrated by centrifugation at 1000 g for 10 minutes. The supernatant was poured off and the cells resuspended in 5 ml of media. An equal volume of 15% DMSO was added dropwise to the cell suspension with constant shaking to give a final DMSO concentration of 7.5%. The tube was held on ice to prevent the trophozoites attaching to the centrifuge tube. The cell suspension was dispensed into 1 ml cryopreservation tubes. These were wrapped in several layers of paper towelling and frozen to -80°C overnight in a polystyrene box. They were then transferred to a liquid nitrogen storage tank. Cryopreserved trophozoites were able to be revived for approximately one year after storage in liquid nitrogen.

2.2.3 Reviving trophozoites from cryopreservation

Materials:

TY1-S-33 growth media, held at 37°C.

Method:

It is necessary to thaw trophozoites held in liquid nitrogen as fast as possible. Tubes containing cryopreserved *G. intestinalis* trophozoites were removed from liquid nitrogen and placed immediately into a 37°C waterbath with the lids loosened. A culture tube was partially filled with pre-warmed medium at this point. Directly the cryopreserved trophozoite solution was thawed, the contents of the tubes were transferred to the culture tube by pipetting slowly under the surface of the medium. The cryopreservation tube was then rinsed with media and the washings transferred to the culture tube. The cell-culture tube was filled to the top with pre-warmed medium and incubated at 37°C for thirty minutes. The media in the tube was dispensed equally into two fresh tubes. All three tubes were topped up with fresh pre-warmed medium and incubated for thirty minutes at 37°C. The medium in the second two tubes was discarded and the tubes refilled with fresh pre-warmed medium. After 3-4 days, high numbers of trophozoites were observed.

2.2.4 Inducing trophozoite cultures to encyst

Materials

TY1-S-33 medium ingredients (see 2.2.1)

Bovine bile (Sigma)

Lactic acid, hemi-calcium salt (Sigma)

Method

The TY1-S-33 medium was made according to the method of section 2.2.1, except that the concentration of bovine bile was increased from 0.8 g/L to 6.1 g/L, and lactic acid added for a final concentration of 0.8 g/L. The pH was then adjusted to 6.8 - 7.0 and the encystation medium filter-sterilised as described in section 2.2.1. Trophozoites in the late log phase of growth were incubated at 37°C in this supplemented medium for up to

three days. The old medium was decanted and the cell-culture tubes filled with the encystation medium, pre-warmed to 37°C. After three days, the contents of the tubes were decanted into sterile 50 ml centrifuge tubes and centrifuged at 1000 g for 5 minutes. The media was decanted off and the tubes filled with milli-Q water and centrifuged for another 5 minutes. This washing process was repeated twice. The cyst/trophozoite pellet was resuspended in 5 ml of milli-Q water.

2.3 Measuring the viability of cysts

2.3.1 Excystation of *G. intestinalis* cysts

(based on the method of Isaac-Renton *et al.*, 1992 and Boucher and Gillin, 1990)

Materials

$5X10^4$ to 10^5 cysts

0.85 g NaCl in 100ml distilled water with 0.7 ml conc HCl, final pH 1.4

20 ml 1X HBSS with 0.1 g L-cysteine (57 mM) and 0.4 g glutathione (32 mM)

0.1 M $NaHCO_3$ - 0.42 g $NaHCO_3$ in 50 ml water

Tyrodes Salt Solution (TSS)

α-chymotrypsin(Sigma), 1 mg per ml in TSS, 10 ml

TY1-S-33

Method

The acidified saline solution, the supplemented HBSS and the sodium bicarbonate solution were made in advance in well-washed glass bottles and pre-warmed to 37°C. Both the HBSS solution and the sodium bicarbonate solution were made up fresh every day. The cysts were pipetted into a 15 ml centrifuge tube and centrifuged at 1000 g for five minutes. The supernatant was drawn off and discarded. To carry out the acid-induction reaction, 5 ml of HCl-saline, 2.5 ml of supplemented HBSS and 2.5 ml of 0.1 $NaHCO_3$ solution added to the cysts, in that order. The centrifuge tube was immediately tightly capped and vortexed. The solution was incubated for 30 minutes in a 37°C waterbath. On removal, it was centrifuged for 5 minutes at 1000 g, and the supernatant

was drawn off. Next, 2 ml of α-chymotrypsin in TSS solution was added to the cysts, which were then centrifuged at 350 g for 5 minutes and the supernatant drawn off. Another 0.5 ml of α-chymotrypsin-TSS solution was added to the cysts, which were mixed by pipetting up and down once. The cysts were incubated for one hour at 37°C. They were centrifuged at 1000 g for 5 minutes, then the supernatant removed. The pellet was resuspended in 2 ml of TSS, centrifuged at 1000 g for 5 minutes, and the supernatant discarded. The pellet was then resuspended in 200 µL of TY1-S-33 medium and transferred to a 250 µL tube. This was kept tightly capped at 37°C. Aliquots of 10 µL were removed and placed onto glass slides. The excysted cysts were viewed under phase contrast microscopy at 400X magnification, and the excystation percentage calculated according to the method of Sauch (1988):

$$\% \text{ excystation} = \frac{\text{ECW} + \text{PET}}{\text{ECW} + \text{PET} + \text{IC}} \times \frac{100}{1}$$

where ECW is the number of empty cyst walls
PET is the number of partially excysted trophozoites
IC is the number of intact cysts

Hanks Balanced Salt Solution (HBSS)

Solution A

1. NaCl	160 g
KCl	8 g
$MgSO_4.7H_2O$	2 g
$MgCl_2.6H_2O$	2 g
Distilled water	800 ml
2. $CaCl_2$	2.8 g
Distilled water	100 ml

Mix solutions 1 and 2 and make up to a final volume of 1L with distilled water. Add 2 ml of chloroform and store at 4°C.

Solution B:

$Na_2HPO_4.12H_2O$	3.04 g
KH_2PO_4	1.2 g
Glucose	20.0 g
Distilled water	800 ml

Mix solution B with 100 ml of 0.4% phenol red in NaOH and make up to 1 L with distilled water. Add 2 ml of chloroform and store at 4°C.

To make up working strength HBSS:

Solution A	100 ml
Solution B	100 ml
Distilled H_2O	800 ml

Final volume is 1 L. This solution can be sterilised by membrane filtration or by autoclaving at 121°C for 5 minutes. For excystaion reactions, the HBSS is not required to be sterile.

Tyrodes Salt Solution

$CaCl_2$	0.265 g
$MgCl_2$-$6H_2O$	0.214 g
KCl	0.2 g
$NaHCO_3$	1.0 g
NaCl	8.0 g
Na_2HPO_4 (monobasic)	0.05 g
Glucose	1.0 g
Distilled water to	1 L

Store in 50 ml aliquots at -20°C.

2.3.2 Use of fluorogenic dyes to ascertain viability of cysts

(based on the method of Campbell *et al.*, 1992)

Materials

4',6-diamidino-2-phenylindole (DAPI) working solution

Propidium Iodide (PI) working solution

Giardia cyst suspension, 10^5 cysts/ml

Milli-Q water

Method

From the well-mixed cyst suspension 100 μL was pipetted into a 1.5 ml Eppendorf tube. 10 μL of DAPI working solution and 10 μL of PI was added, and the solution mixed thoroughly. If a fluorescein-labelled monoclonal antibody is required, 10 μL can be added at this point. The mixture was then incubated at 37°C for ten minutes. The suspension was immediately washed by adding 1 ml of purified water, centrifuging for 5 minutes at 1000 g and drawing off the supernatant. This washing step was repeated twice. After the final wash, enough of the supernatant was drawn off to leave 50-100 μL of suspension remaining in the tube. After mixing the suspension, 10 μL was drawn off and placed on a glass slide. A cover slip was placed over the top, and the edges of the slip sealed down with nail polish. The cysts were viewed under a Reichart-Jung epifluorescent microscope. The DAPI staining behaviour was observed under the UV filter block, at an excitation wavelength of 340-380 nm. The propidium iodide staining pattern was observed under a green filter with an excitation wavelength of 515-560 nm. The monoclonal antibody stained cysts were observed under a blue filter with an excitation wavelength of 450-490 nm. The cysts can be located under the blue filter, then the state of their viability seen using the UV and the green filter. Where it was confident that the cyst solution contained few other organisms, it was more convenient to locate cysts using the UV filter. The percentage of viable cysts was then calculated. For the experiments carried out, 400-500 cysts were counted at each point.

Table 1. Correlation of *Giardia intestinalis* cyst viability with inclusion of DAPI and PI

Cyst type	DAPI inclusion	PI inclusion	Viability
Ghost	None	None	Dead
PI+	Yes	Yes	Dead
DAPI+/PI+	Yes	Yes	Dead
DAPI+/PI-	Yes	None	Viable
DAPI-/PI-	None	None	Viable

4',6-diamidino-2-phenylindole (DAPI) working solution

2 mg of DAPI powder was dissolved in 1 ml of absolute methanol. This was stored at 4°C in the dark.

Propidium iodide (PI) working solution

1 mg of propidium iodide powder was dissolved in 1 ml of 50 mM PBS (pH7.2). This was stored at 4°C in the dark.

2.4 Preparation of *in vivo* trophozoites for electron microscope photography

Materials:

Infected hamsters (or other rodents)

Phosphate buffered saline (PBS) solution

EM fixer buffer (from the Electron Microscope Unit at HortResearch)

Method:

Hamsters carrying *Giardia intestinalis* were initially identified and killed by exposure to CO_2. Using a scalpel, a slit was made in the skin of the hamster from the lower intestinal region up to the lung cavity. The small intestine was isolated and snipped free at either end. This was slit lengthwise along the entire length of the intestine. The intestine was then placed in a 50 ml centrifuge tube partially filled with phosphate buffered saline (PBS) which was then vortexed. The centrifuge tube was topped up to 50 ml with PBS and placed in ice.

After 10-15 minutes, the tube was shaken and the supernatant poured into small glass petri dishes, which were examined under a light microscope for the presence of trophozoites. These petri dishes contained small coverslips which covered the bottom surface. The Centrifuge tube was topped up with PBS and again placed into ice for 10-15 minutes. The supernatant was again poured off into petri dishes and examined. This process of cooling and pouring off was repeated four times. The petri dishes containing coverslips and supernatant were incubated at 37°C.

After 30 minutes, the petri dishes were examined under light microscope to ascertain whether the trophozoites had attached to the surface. If the numbers observed seemed low, the petri dishes were incubated for a further 30 minutes. Once high concentrations of trophozoites had attached, the coverslips were removed with tweezers and dipped into PBS warmed to 37°C. This was repeated four times in order to rinse off extraneous material. They were then placed into small glass Kimax tubes containing EM fixer. Where a mixed specimen of *Giardia* from the hamster and *G. intestinalis* from a culture were required, the coverslips were removed from the petri dishes and placed face-up into fresh petri dishes. The *G. intestinalis* trophozoite culture in growth medium was then poured into the petri dish, and left for 30 minutes to allow the trophozoites to attach to the coverslips. The 3-day old cultures had sufficient floating trophozoites to attach to the coverslips without the need to chill additional trophozoites off the surface of the cell culture tube. Once attached, these coverslips too were dipped in warmed PBS and stored in EM fixer.

Phosphate Buffered Saline (PBS) pH 7.2

NaCl	8.5 g	34.0 g	42.5 g
$Na_2HPO_4.12H_2O$	2.7 g	10.8 g	13.5 g
$NaH_2PO_4.2H_2O$	0.39 g	1.56 g	1.95 g
Distilled H_2O	1 L	4 L	5 L

1 M HCl or 1 M NaOH to pH 7.2

Autoclave at 121°C for 15 minutes.

2.5 DNA extraction

2.5.1 DNA extraction from *Giardia* trophozoites

Materials

G. intestinalis culture

10% Sodium Dodecyl Sulphate (SDS)

Pronase type XIV (Sigma) 10 mg/ml in distilled water

RNase (Ribonuclease I) (Sigma) 2 mg/ml in distilled water

5 M Sodium Perchlorate

10X Saline Tris-EDTA (STE) Buffer

Phenol/Chloroform/Isoamyl alcohol

Tris-EDTA (TE) buffer

Method

Trophozoites from a culture in the exponential stage of growth (2-3 days old) were pelleted by centrifugation at 500 g for 5 minutes. When not processed immediately, these were stored in 5 ml of TE buffer at -20°C. The pellet was resuspended in 1 ml of TE buffer. Sufficient 10% SDS was added to the cell suspension to bring the concentration to 1% SDS in the sample (eg 100 µL for a 1 ml sample). Pronase type XIV was also added for a final concentration of 1 mg/ml (eg 100µL of a 10 mg/ml solution to a 1 ml sample). This suspension was incubated overnight at 50°C.

The next day, 0.1 ml of 2 mg/ml RNase was added to the lysate and incubated for 60 minutes at 50°C. 5 M sodium perchlorate was added for a final concentration of 1 M, in this case 260 µL. The lysate was then incubated at 50°C for another 60 minutes. While this was incubating, phenol was added to chloroform and isoamyl alcohol at a ratio of 25:24:1 and one tenth of the volume added in STE buffer. Air was bubbled through the solution to aid mixing and then left to cool to room temperature. An equal volume of the phenol/chloroform/isoamyl alcohol was added to the lysate, mixed by inverting several times and left to stand for 5 minutes. The mixture was then centrifuged at 5000 g for 10 minutes. The upper aqueous phase containing the DNA was drawn off and the process repeated until white precipitate was no longer visible at the interface.

One-twentieth of the volume in 5 M NaCl and 2 volumes of absolute ethanol at -20°C was then added and the DNA precipitated at -20°C overnight. The mixture was centrifuged at 16000 g for 30 minutes to pellet the DNA and the supernatant drawn off. The remainder was washed with absolute ethanol, dried and resuspended in TE buffer.

1.0 M Tris-HCl pH 7.5

Trizma base (Sigma) 121.1 g
Distilled water to 1 L
Adjust to pH 7.5 using 5 M HCl

0.2 M EDTA pH 7.2

Ethylamine diamine tetra-acetic acid (disodium salt) 7.44 g
Distilled water to 1 L
Adjust to pH 7.2 using 5 M NaOH

Tris-EDTA (TE) buffer

1.0M Tris-HCl (pH 7.5)	1.0 ml
0.2M EDTA (pH 7.2)	5.0 ml
Distilled water to	10.0 ml

Autoclave at 121°C for 15 minutes, and store at room temperature.

Saline Tris-EDTA (STE) buffer

5.0 M NaCl	20 ml
1.0 M Tris-HCl (pH 7.5)	50 ml
0.2 M EDTA (pH 7.2)	5 ml
Distilled water to	100 ml

Autoclave at 121°C for 15 minutes, and store at room temperature.

RNase

2 mg/ml in distilled water. This solution is preincubated at 90°C for 10 minutes to destroy DNase activity and stored at -20°C.

2.5.2 DNA extraction from *Giardia* cysts

Materials:

Giardia intestinalis cysts extracted from faecal matter (see method 2.1)

Chelex solution -

10% w/v Chelex 100 (Bio-Rad)

0.1% w/v SDS (sodium dodecyl sulphate) solution

1% v/v Nonidet P40

1% v/v Tween 20

G. intestinalis cysts extracted from faecal matter (see method 2.1)

Method

A volume of the cyst solution containing at least 10 cysts was centrifuged at 2000 g to pellet the cysts. The supernatant was drawn off and the cysts resuspended in 100 µL of Chelex solution, ensuring the Chelex solution was well-mixed immediately prior to dispensing. This was incubated at 100°C for thirty minutes. The suspension was centrifuged at 14 500 g for five minutes and the supernatant transferred to a new tube. The supernatant containing the DNA was left to stand for at least two hours before use in PCR reactions

2.6 DNA amplification using the Polymerase Chain Reaction (PCR)

Materials

10X DNA Taq polymerase buffer (Gibco-BRL)

$MgCl_2$ (Gibco-BRL) - 50 mM

dNTPs (Gibco-BRL) - 1.25 mM each of dATP, dTTP, dGTP and dCTP

PCR primers (Amrad) - 1 nmol/μL in sterile distilled water

Taq Polymerase (Gibco-BRL) - 5U/μL

Glycerol (BDH) Molecular biology grade

Dimethyl sulphoxide (DMSO)

Sterile milli-Q water

Agarose (BioRad) Analytical grade

1X E-Buffer

Method

The basic PCR mixture was comprised of:

1X PCR buffer (consisting of 20 mM Tris-HCl and 50 mM KCl), 1.5 mM $MgCl_2$, 250 μmol of each dNTP, 10 pmol each of primer 1 and primer 2, 20 - 200 ng of template DNA, 1 unit of Taq polymerase, and sufficient sterile water to bring the total reaction volume to 20 μL, or whatever volume was required. Glycerol, at a concentration of 10%, was required in reactions for some primers, while the presence of DMSO was necessary for reactions using other primers.

The PCR amplifications were carried out using a Perkin Elmer Cetus DNA Thermal Cycler (9600). The initial programme in the method consisted of one cycle - a 5 minute denaturing step at 95°C, a 30 second annealing step at 50°C, and a 30 second extension step at 72°C. The next programme was 45 cycles - 30 seconds at 95°C, 30 seconds at 50°C and 30 seconds at 72°C. The final programme was again one cycle - 30 seconds at 95°C, 30 seconds at 50°C and 5 minutes at 72°C. The reaction was held at 4°C. The annealing temperatures varied according to the primers in the reaction. As the rDNA

The PCR products were analysed by gel electrophoresis, using 1.6% agarose in 1X E-buffer run at 100 V for one hour in a BRL Horizon 54 gel box. An aliquot of 2 μL of PCR sample was mixed with 8 μL of 1X bromophenol blue dye, and loaded onto the gel. The gel was stained in ethidium bromide (5 μg/ml) for 10 minutes and destained in milli-Q water for 10 minutes. It was then viewed and photographed under UV light.

Bromophenol blue dye (10X)

0.1% w:v bromophenol blue

80% glycerol v:v

This was made up in distilled water, and for use diluted 1:10 in 1X E-buffer.

10X E-buffer

Trizma	96.88g
EDTA (disodium salt)	7.44g
Sodium acetate	8.2g
Distilled water to	2L

Adjust pH to 7.8 using acetic acid. For use dilute one in ten.

2.7 PCR primers for *Giardia intestinalis* rDNA

2.7.1 *G. intestinalis* rDNA PCR primer sequences

(developed from sequences in Healy *et al.*, 1990)

16Sr-1	5'- ATC CGG TCG ATC CTG CCG GAG CG -3'
16Sr-2	5'- GAT CCA TCC GCA GGT TCA CCT ACG -3'
Uno-1	5'- CGG GTG AAA CAG GAT CCC -3'
Uno-2	5'- CGA CTT CTC CTT CCT CCA GGC -3'
Joli-1	5'- CGA TAG CAG GTC TGT GAT GC -3'
Joli-2	5'- CGT ACT GAT ATG CTT AAG TTC -3'

Jay-1	5'- CTT AAG CAT ATC AGT ACG CCC C -3'
Jay-2	5'- TTG TTA CAC ACT CCC TGG AGG -3'
Koni-1	5'- CGA AAG GCG GTG ATC TAT GCC -3'
Koni-2	5'- CGA CGA GGC ATT TGG CTA CC -3'
Ace-1	5'- GGA GTG TGT AAC AAC CCA CCA -3'
Ace-2	5'- GCG GCC ACA AGC CAG TTA TCC -3'
Thor-1	5'- GAA TGG ACC AAC GAG GAT CCC -3'
Thor-2	5'- CTA ACC TGT CTC ACG ACG GTC -3'

2.7.2 PCR Primer Combination Sizes

Uno-1 combinations

Uno-1/Uno-2	773bp
Uno-1/Joli-1	1089bp
Uno-1/Jay-2	2010bp
Uno-1/Koni-2	2815bp
Uno-1/Ace-2	3293bp
Uno-1/Thor-2	3434bp

Joli-1 combinations

Joli-1/Joli-2	616bp
Joli-1/Uno-2	289bp
Joli-1/Jay-2	1537bp
Joli-1/Koni-2	2342bp
Joli-1/Ace-2	2820bp
Joli-1/Thor-2	2961bp

Jay-1 combinations

Jay-1/Jay-2	938bp
Jay-1/Joli-2	17bp
Jay-1/Koni-2	1743bp
Jay-1/Ace-2	2221bp
Jay-1/Thor-s	3262bp

Koni-1 combinations

Koni-1/Koni-2	1258bp
Koni-1/Jay-2	453bp
Koni-1/Ace-2	1736bp
Koni-1/Thor-2	1877bp

Ace-1 combinations

Ace-1/Ace-2	1296bp
Ace-1/Jay-2	13bp
Ace-1/Koni-2	818bp
Ace-1/Thor-2	1437bp

Thor-1 combinations

Thor-1/Thor-2	599bp
Thor-1/Ace-2	438bp

2.8 Sequencing from PCR samples

Materials

AmpliCycle Sequencing Kit (Perkin Elmer)

A, T, G and C – termination mixes

'Stop' solution

10 X cycling mix

PCR template

PCR primers corresponding to template (Amrad) – 1 nmol/μL in sterile distilled water

α-33P (Amersham), 10 Ci/ml

Method

A standard cocktail was put together using 1 μL of Primer 1 or 2, 1 μL of 33P, 4 μL of 10X cycling mix and 1 μL of PCR template. Sterile water was added to bring the total volume to 30 μL. Aliquots of 2 μL of A-, T-, G- and C-termination mixes were

Method

A standard cocktail was put together using 1 μL of Primer 1 or 2, 1 μL of 33P, 4 μL of 10X cycling mix and 1 μL of PCR template. Sterile water was added to bring the total volume to 30 μL. Aliquots of 2 μL of A-, T-, G- and C-termination mixes were dispensed into 4 separate PCR tubes, and 6 μL of the standard cocktail added. These were held on ice until processed. All four mixes underwent a programme on the Perkin Elmer Cetus DNA Thermal Cycler (9600) of: 95°C for 60 seconds; then 25 cycles of 95°C for thirty seconds, 68°C for 30 seconds and 72°C for 60 seconds. Finally they were held at 4°C (less than 40 minutes). 4 μL of 'stop'solution were added to each tube.

The sequence of the Joli-fragment was analysed by hand using a 6% acrylamide sequencing gel at a constant voltage of 1500 V, with a short run time of 2 hours and a long run of 4 hours.

The Joli-fragment PCR sample was also sequenced automatically using an ABI Prism 377 Genetic Analyzer.

40% Acrylamide stock

Acrylamide	76 g
Bis-acrylamide	4 g
Distilled water to	200 ml

Store at 4°C for up to 2 months

10X Sequencing buffer

1.35 M Tris-HCl	163.35 g
450 mM Boric acid	27.8 g
25 mM EDTA	9.3 g
Distilled water to	1 L

pH should be 8.8

Urea/Acrylamide/Buffer mix

8M Urea	288 g
40% Acrylamide/bis-acrylamide	90 ml
OR: acrylamide 34.2 g	
bis-acrylamide 1.8 g	
Distilled water to	540 ml

Warm the solution, but to no hotter than 40°C otherwise the urea will break down. Filter using a scintered glass funnel or Whatman filter paper. Add 60 ml of 10X sequencing buffer. Store in plastic at room temperature or at 4°C, but be careful of urea crystals forming at this temperature and stand at room temperature before use. This volume is sufficient for 6 gels and can be stored for up to 1 month.

6% Acrylamide/bis-acrylamide gel

Urea/acrylamide/buffer mix	90 ml
10% Ammonium persulphate	600 µL
TEMED	60 µL

Mix well and pour.

Chapter 3. Results

3.1 Generation of *in vitro Giardia intestinalis* cysts from trophozoite culture

Table 2: Numbers of *in vitro Giardia intestinalis* cysts recovered over time in encystation media

Days incubation	Organisms/ml
0	7.2×10^5 trophozoites/ml
1	6.8×10^4 cysts/ml
2	1.9×10^5 cysts/ml
3	3.0×10^5 cysts/ml
4	3.5×10^5 cysts/ml

The yield of *Giardia intestinalis* cysts after an incubation period of one day in encysting medium was lower than for days 2-4, but the morphology of the cysts viewed under phase contrast microscopy closely approximated the appearance of fresh, viable cysts. The cysts were smooth and hyaline, with no internal structures visible. After two days' incubation in encysting medium, the cysts still displayed the morphology of viable cysts, with a yield of 1.9×10^5 cysts/ml of encystment media. By the third day, although the cyst yield was higher at 3.0×10^5 cysts/ml of media, the morphology of the cysts had changed, so that the internal structures were visible, concentrated in the centre of the cyst. The increase in cyst numbers between days 3 and 4 was quite low.

3.2 Use of DAPI to measure the viability of *in vivo Giardia intestinalis* cysts

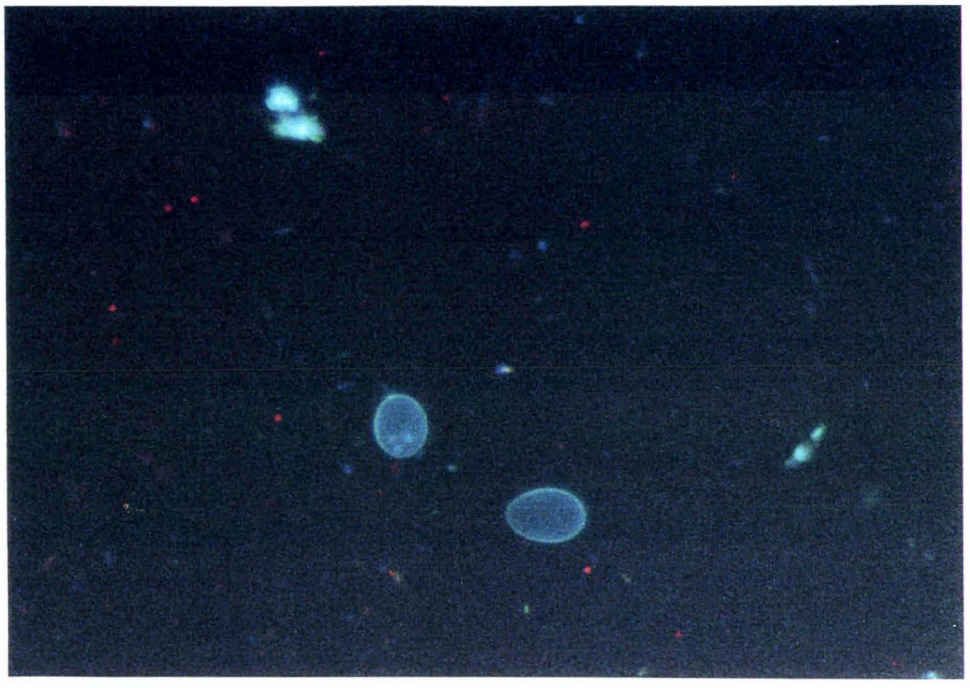
Table 3. The change in proportion of DAPI-/PI- staining cysts over time

	30 min stain	1 hour stain	90 min stain	2 hour stain
(1) Ghosts	0%	0%	0%	0%
(2) PI +	14.3%	29%	75%	89%
(3) DAPI+/PI-	3.6%	18.1%	10.6%	3.9%
(4) DAPI-/PI-	82.1%	52.9%	14.4%	7.1%
Viable cysts (3) + (4)	**85.7%**	71%	25%	11%

The time for which the cysts were incubated with the stains was extended to examine if this would increase the number of cysts whose nuclei stained with DAPI. The number of PI+, or nonviable, cysts rose from 14.3% after a 30 minute stain time to 89% after a 2 hour stain time. On the other hand the number of DAPI+/PI- cysts, those whose nuclei were stained with DAPI, also increased from 3.6% after a 30 minute stain time to 18.1% after a 60 minute stain time. It would seem that the DAPI-/PI- cysts are viable cysts which require a longer stain time in order for the nuclei to become stained. In addition, the viability for the sample of cysts as measured by excystation was 88%. This ties in well with the combined number of DAPI+/PI- cysts and DAPI-/PI- cysts (85.7%) seen after a stain time of 30 minutes.

Plate 1: *Giardia intestinalis* cysts stained with DAPI and PI viewed under UV-filter. 400X magnification.

A: DAPI+ (left) and DAPI- (right) viable cysts.



B: Comparison of viable and non-viable cysts: DAPI- viable cysts (blue outline) and DAPI+ non-viable cysts (pink cytoplasm).

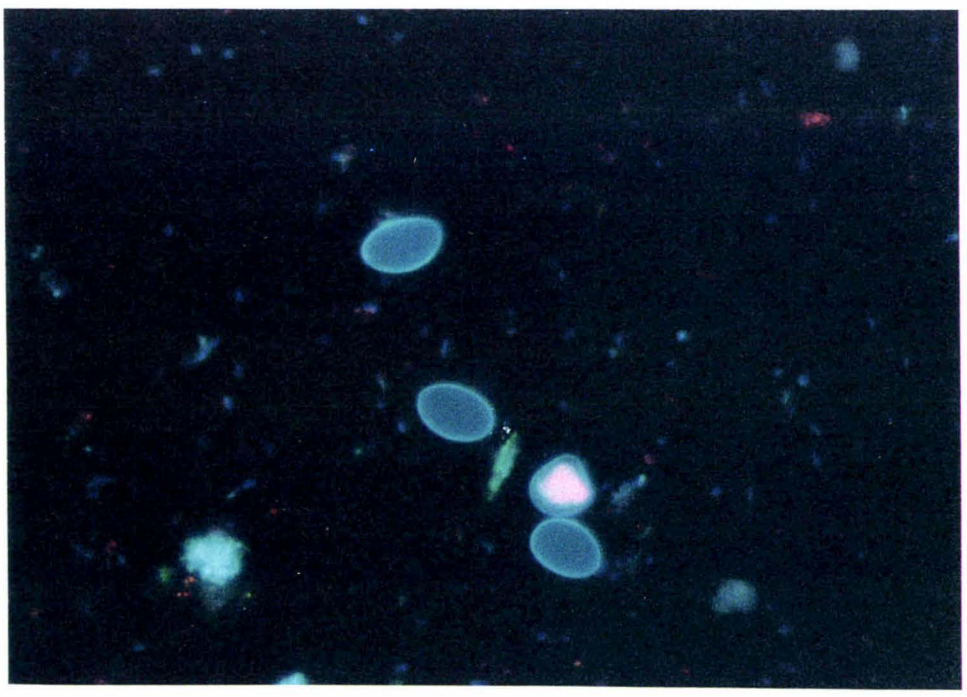
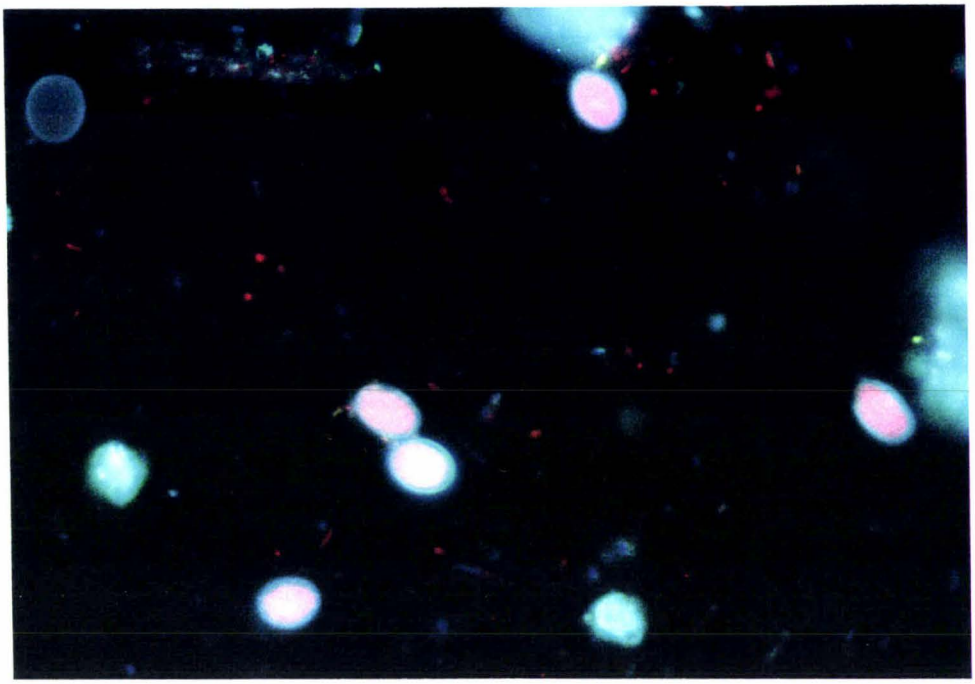
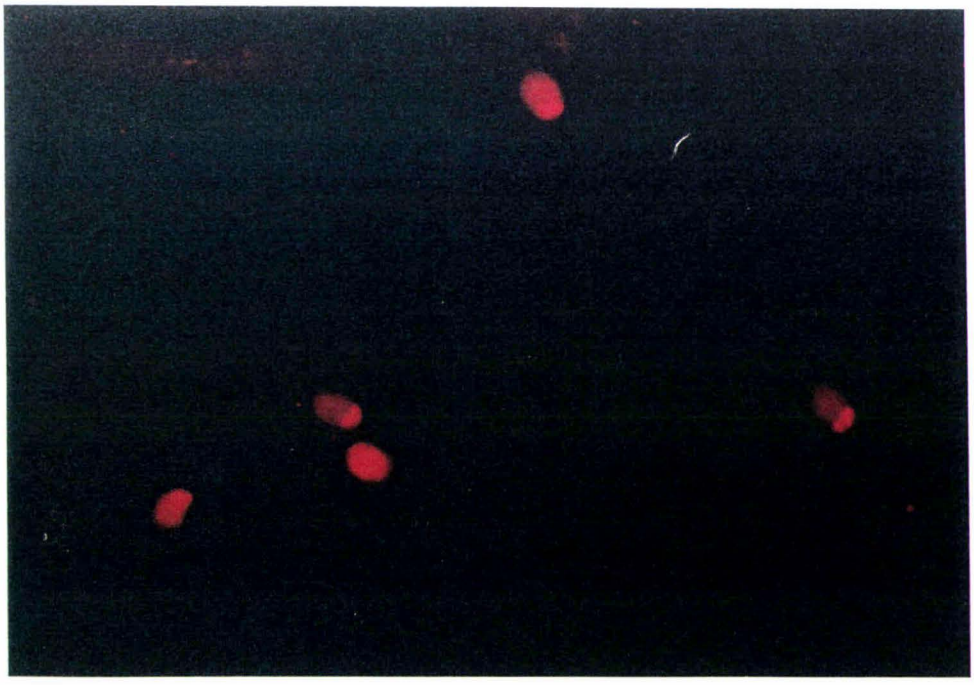


Plate 2: Non-viable *Giardia intestinalis* cysts stained with DAPI and PI. 400X magnification.

A: DAPI+ non-viable cysts viewed under UV filter.



B: PI+ non-viable cysts (same frame as for 'A' above) viewed under green filter.



3.3 Viability of *Giardia intestinalis* cysts

Fig 1. Viability of *Giardia intestinalis* cysts isolated from human faeces at 15C in sea water and milli-Q water, as measured by excystation

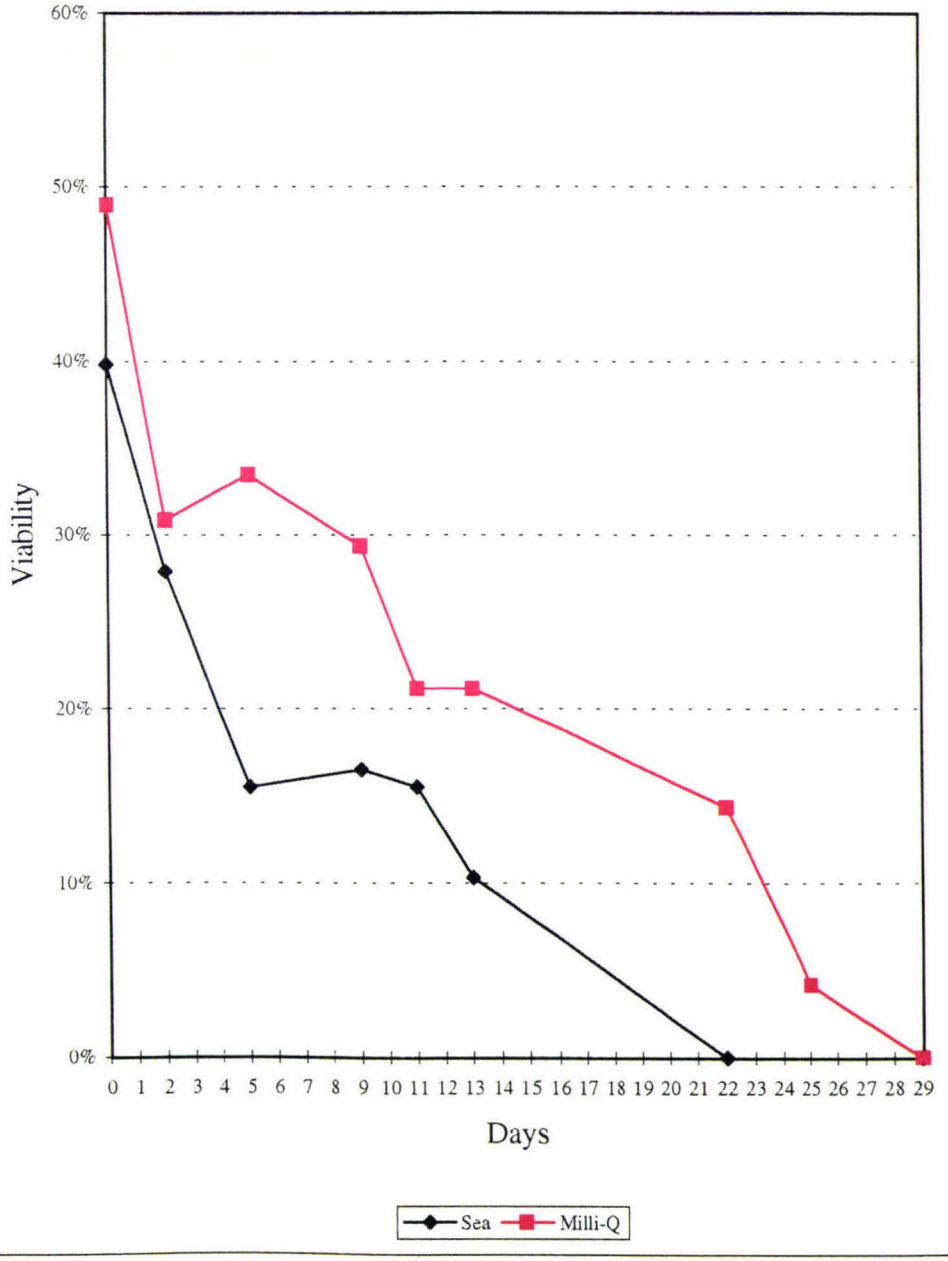


Fig. 2 Viability of *Giardia intestinalis* cysts isolated from calf faeces at 4C, 10C, 15C, 20C and 25C in milli-Q water, as measured by the fluorogenic dyes DAPI and PI

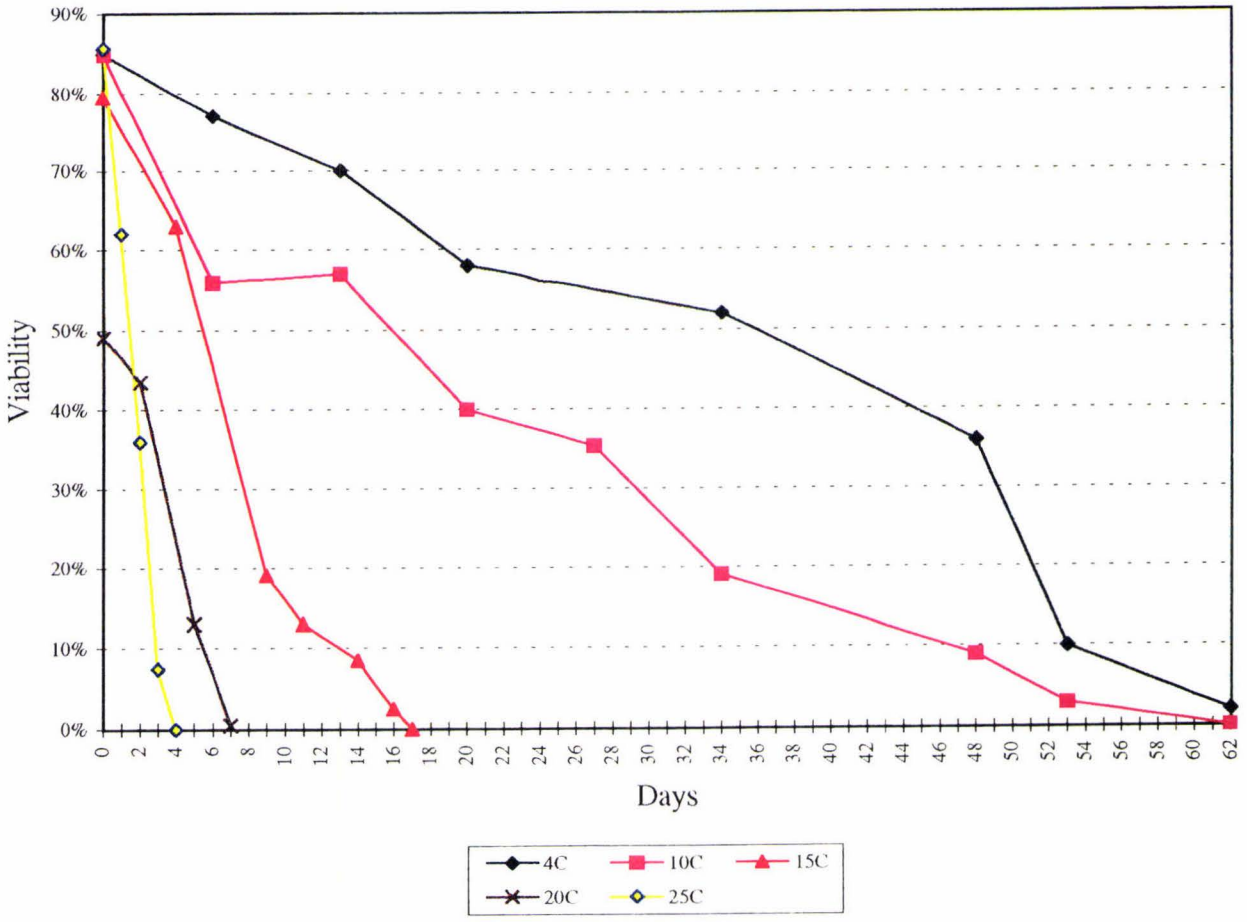
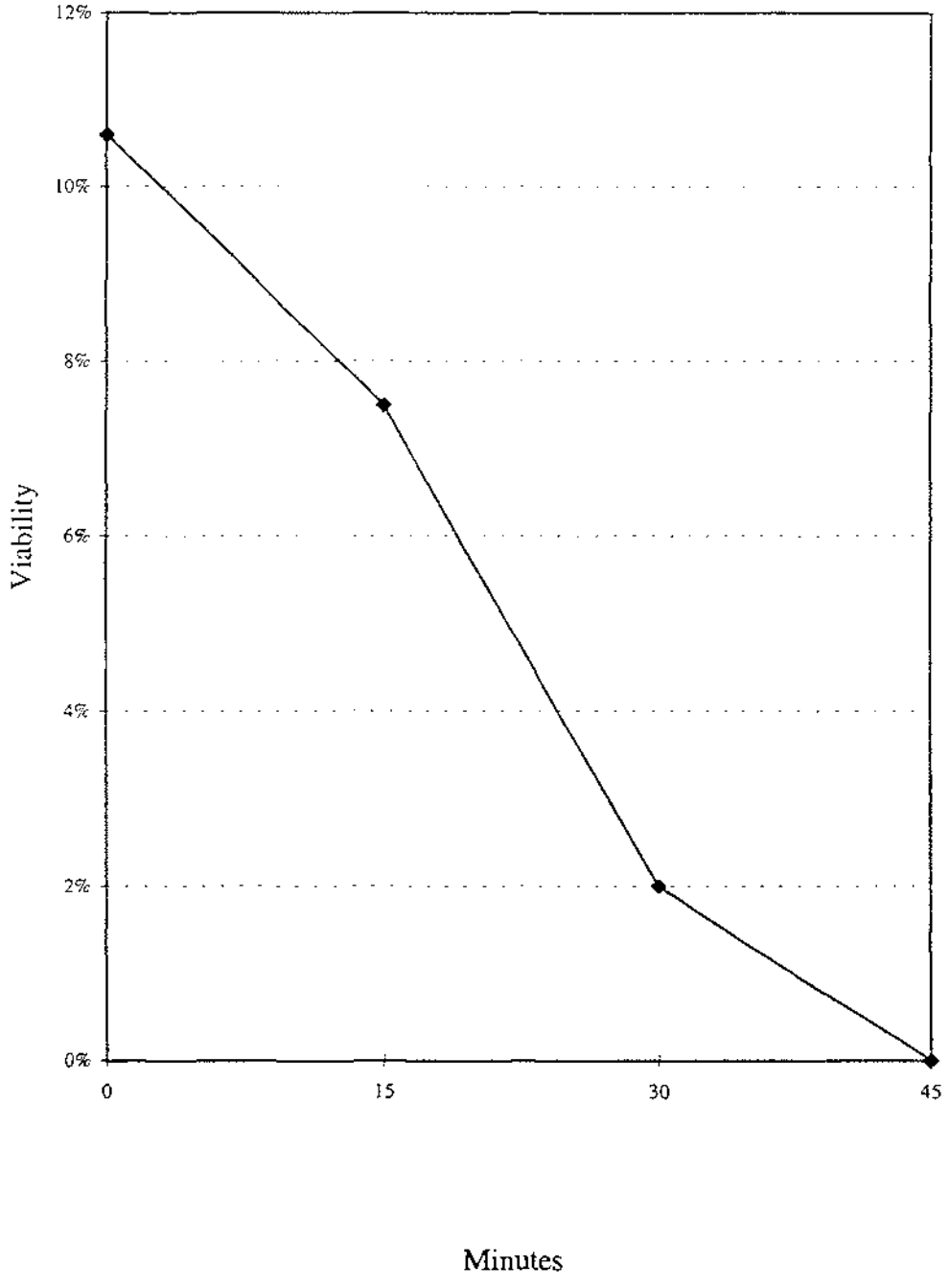


Fig. 3 Viability of *Giardia intestinalis* cysts isolated from calf faeces at 4C in sea water, as measured by the fluorogenic dyes DAPI and PI .

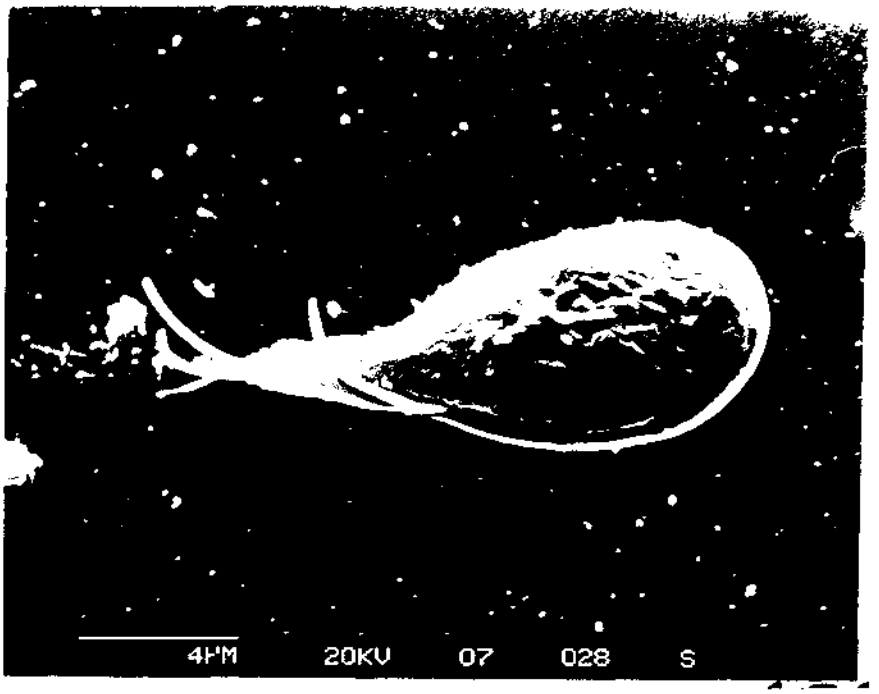
On immersion in the sea water, the viability of the cysts immediately dropped from 54% to 10.6%.



3.4 Analysis of Hamster *Giardia* rDNA

Plate 3: Scanning electron micrographs of the dorsal view of *Giardia* trophozoites with the flagellae and the edge of the ventral sucking disc visible.

A: *Giardia intestinalis* trophozoite from an *in vitro* culture.



B: *Giardia* trophozoite isolated from the small intestine of a hamster.

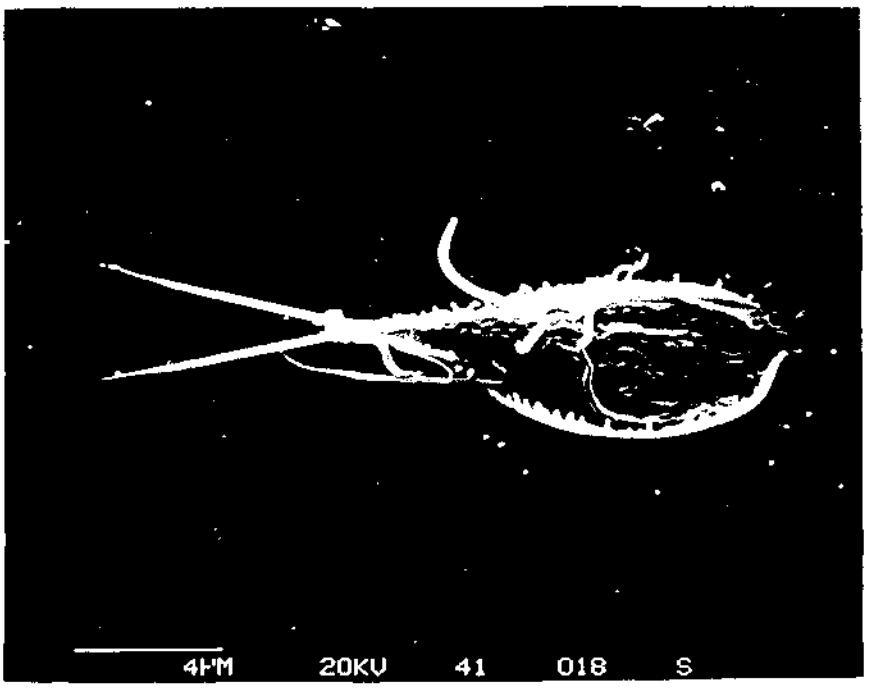
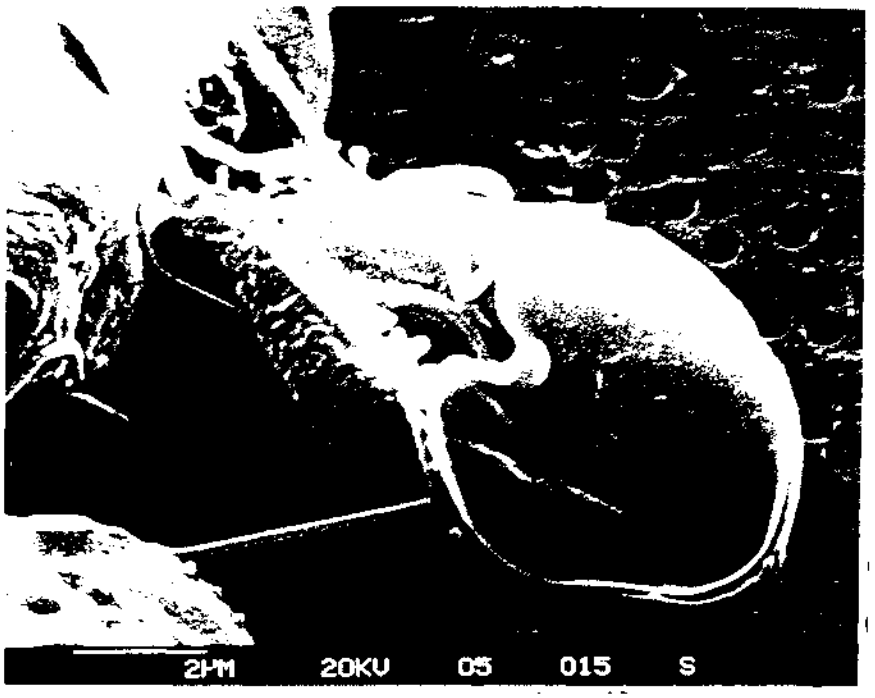


Plate 4: Scanning electron micrographs of *Giardia* trophozoites with the ventral adhesive discs and the ventral flagellae visible.

A: *Giardia intestinalis* trophozoite from an *in vitro* culture



B: *Giardia* trophozoite isolated from the small intestine of a hamster.

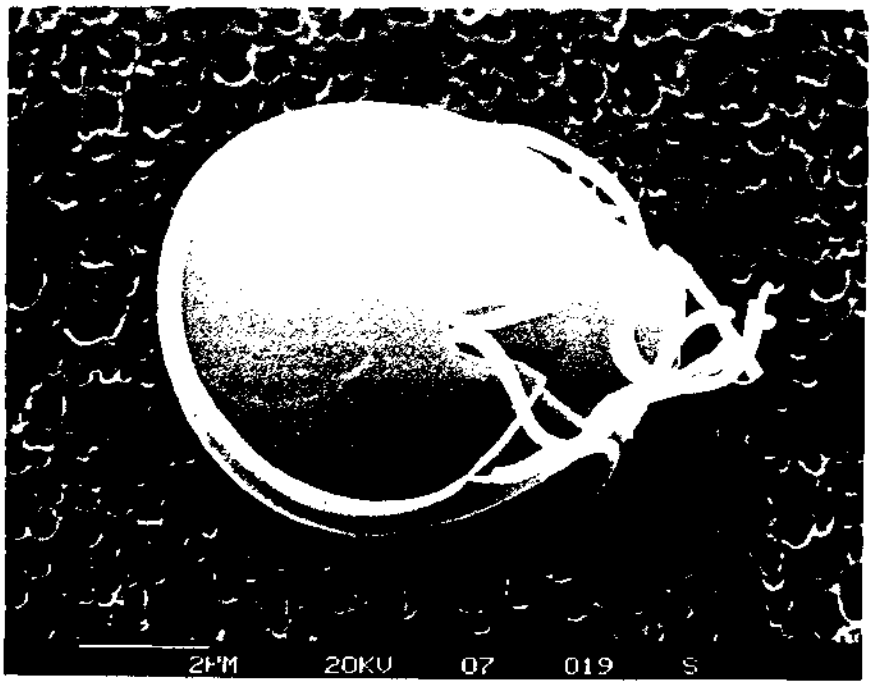


Fig. 4 Map showing for which areas of the rDNA PCR products were obtained for *G. intestinalis*, *G. muris* and hamster *Giardia*.

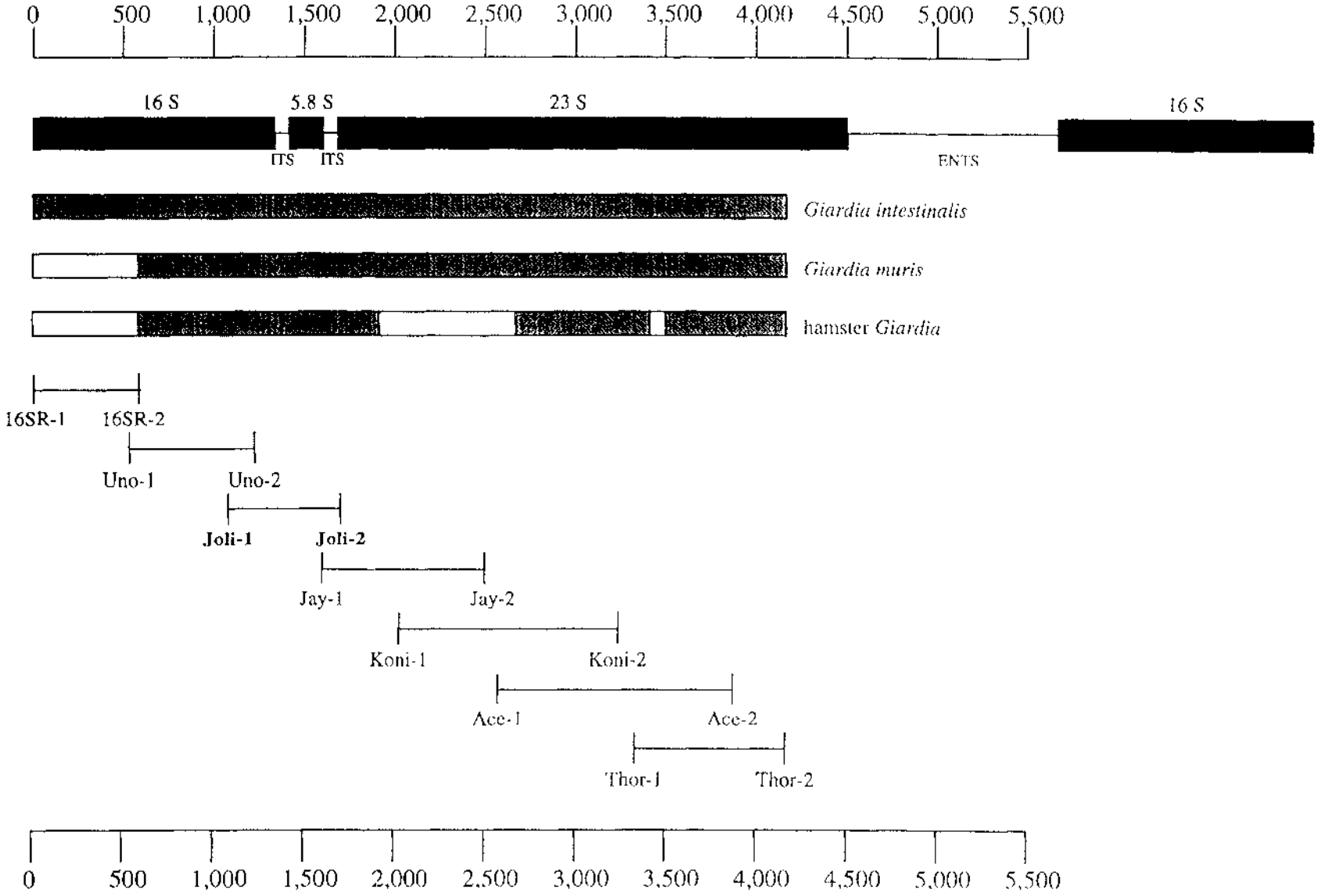
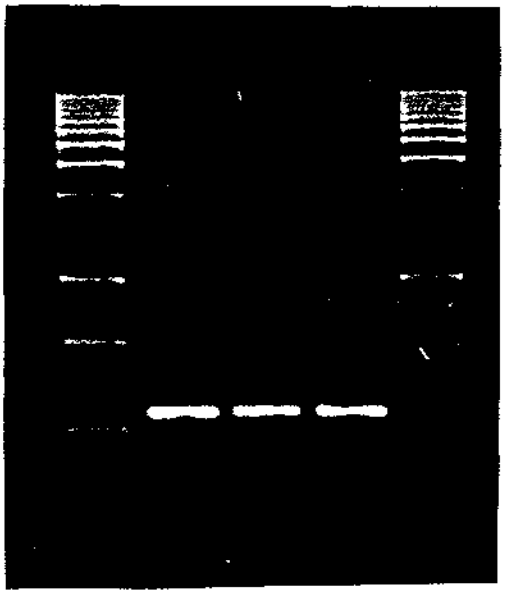


Table 4. Results of PCR reactions using primers designed for *Giardia* rDNA.

A '+' indicates where a fragment was successfully amplified, a '-' where no product was obtained.

Primer pairs	*G. intestinalis*	*G. muris*	Hamster *Giardia*
16SR1 – 16SR2	+	-	-
Uno-1 – Uno-2	+	+	+
Uno-1 – Joli-2	-	-	-
Joli-1 – Joli-2	+	+	+
Joli-1 – Jay-2	-	-	-
Joli-1 – Koni-2	-	-	-
Joli-1 – Thor-2	+	+	-
Jay-1 – Jay-2	+	+	-
Jay-1 – Koni-2	-	+	-
Jay-1 – Ace-2	-	-	-
Jay-1 – Thor-2	+	+	-
Koni-1 – Koni-2	+	-	-
Koni-1 – Jay-2	-	-	-
Koni-1 – Ace-2	-	-	-
Koni-1 – Thor-2	-	+	-
Ace-1 – Ace-2	-	+	-
Ace-1 – Koni-2	+	+	+
Ace-1 – Thor-2	-	+	-
Thor-1 – Thor-2	+	+	+

Fig. 5 Ethidium bromide-stained agarose gel electrophoresis of the 'Joli'-PCR amplification products for *Giardia intestinalis*, *Giardia muris* and *Giardia* from hamsters. All three appear to be about the correct size of 616 bp.



1 2 3 4 5

Lane 1: 1 Kb DNA Ladder
Lane 2: *Giardia intestinalis*
Lane 3: *Giardia muris*
Lane 4: *Giardia* from hamsters
Lane 5: 1 Kb DNA Ladder

Fig. 6 Print-out from the automatic sequencer of the sequence from the 616-bp 'Joli'-PCR fragment amplified from the hamster isolate of *Giardia.* →

ABI PRISM™

Model 377
Version 3.0
ABI100
Version 3.0

10•Giardia.J1
Loaded the sample onto the sequencer only
Giardia.J1
Lane 10

Signal G:2022 A:3239 T:1588 C:1214
DT4%Ac{A Set-AnyPrimer}
seq matrix ccd 190
Points 1031 to 7248 Base 1: 1031

Tue, 1 Jul 1997 10:42 AM
Mon, 30 Jun 1997 2:04 PM
Spacing: 10.97{10.97}

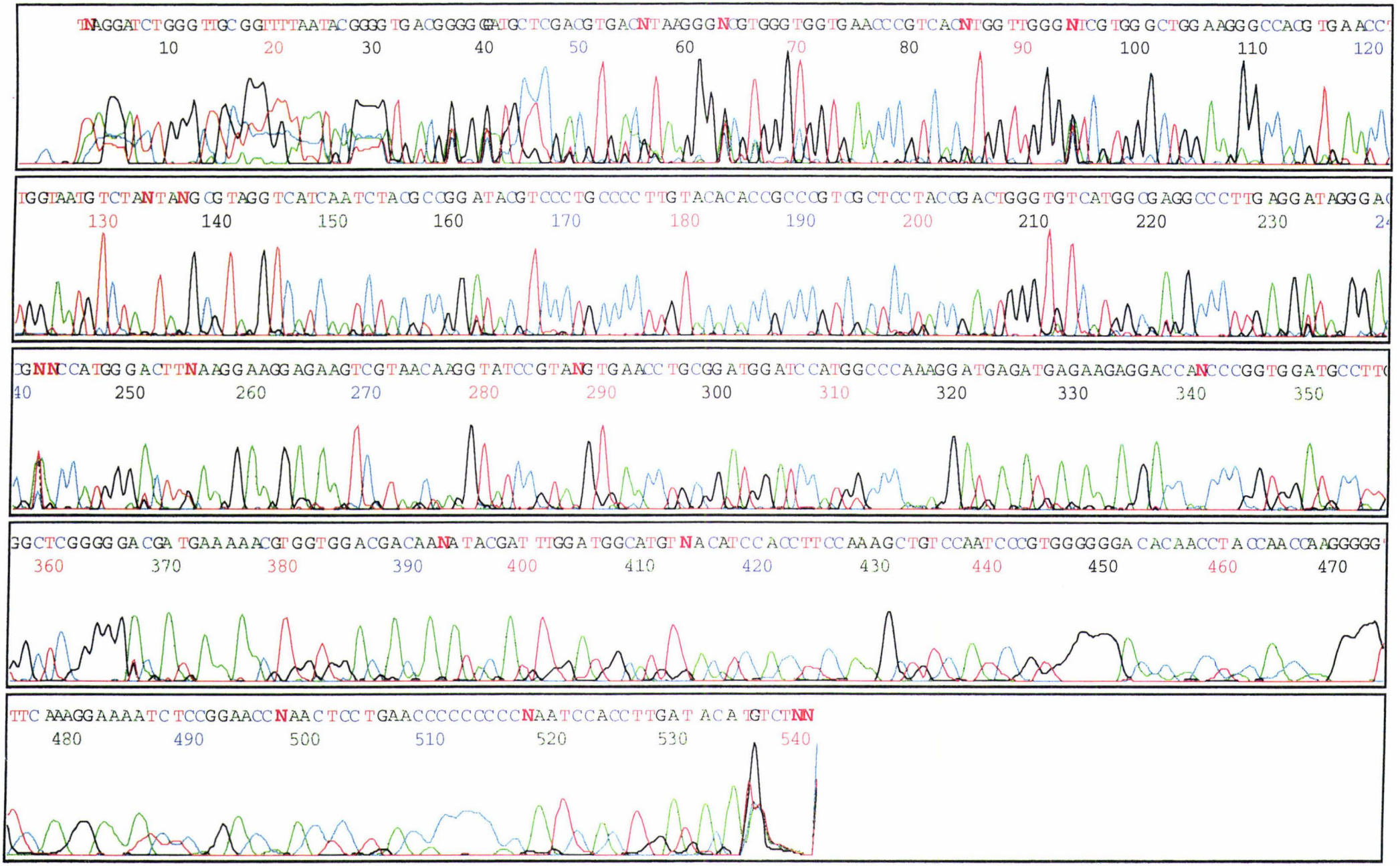
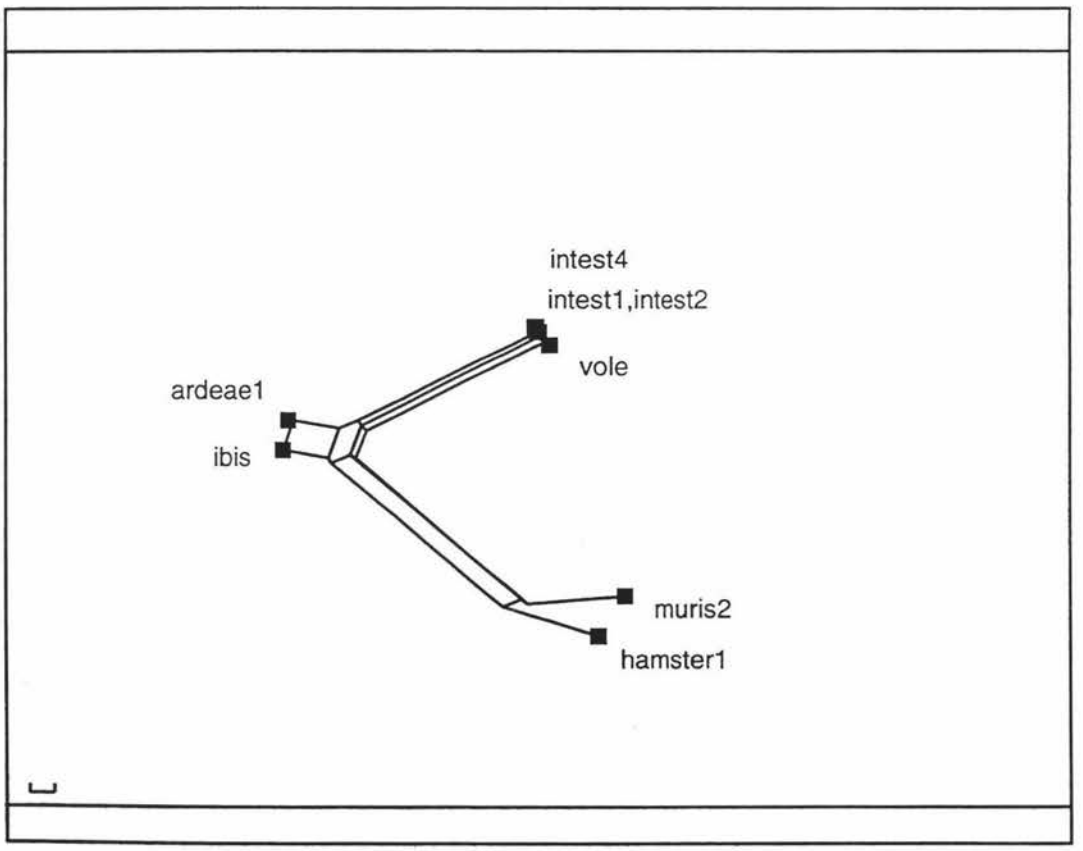


Fig. 7 Comparison of the rDNA sequence from the 'Joli'-PCR fragment amplified from the hamster *Giardia* isolate (hamster1) with published sequences of *Giardia intestinalis* (intest-1, intest2 and intest4), *Giardia ardeae* (ardeae), *Giardia* from ibis' (ibis), *Giardia muris* (muris2), and *Giardia* from voles (vole). The sequences were aligned using CLUSTAL.

```
intest_1        ----------------------GCGAGCGGGAGGACGGCGGGGCGATAGCAGGTCTGTGAT
intest2         ----------------------GCGCGAGCGGGAGGACGGCGGGGCGATAGCAGGTCTGTGAT
intest4         ----------------------GCGAGCGGGAGGACGGCGGGGCGATAGCAGGTCTGTGAT
adreae1         ------------CCGCCGCCGGGGAGGCGGAGGAAGGCGGGGCGATAACAGGTCTGTGAT
ibis            CCGCGTGTGGGACTGCCACTGGTGAAGTGGAGGAAGGCGGGGCGATAACAGGTCTGTGAT
muris2          ----------------------------------------CGATAACAGGTCTGTGAT
vole            ----------------------GCCGAGCGGGAGGAGGGCGGGGCGATAGCAGGCCTGTGAT
hamster1        ------------------------------------------------------------

intest_1        GCCCTCAGACGCCCTGGGCCGCACGCGCGCTACACTGGCGGGGCCAGCCGGCGC----CC
intest2         GCCCTCAGACGCCCTGGGCCGCACGCGCGCTACACTGGCGGGGCCAGCCGGCGC----CC
intest4         GCCCTCAGACGCCCTGGGCCGCACGCGCGCTACACTGGCGGGGCCAGCCGGCGC----GC
adreae1         GCCCTCAGACGCCCTGGGCCGCACGCGTACTACACTGGGCCGGGCAGCGCGCGCGCGGCC
ibis            GCCCTCAGACGCCCTGGGCCGCACGCGTACTACACTGGGCCGGGCAGCGCGCGCGTGTCC
muris2          GCCCTTAGACGCCCTGGGCTGCACGCGTACTACACTGTGGGGATGAAACCACGTC---GA
vole            GCCCTCAGACGCCCTGGGCCGCACGCGCGCTACACTGGCGGGGCCAGCCGGCGC----CC
hamster1        ----------------------------------------GATGCT-CGACGT----GA
                                                          *       **

intest_1        GCGAGGACGCGCGGAGCCCCCG------CCGTGGCCGGGACCGCGGGCTGGAACGCCCCC
intest2         GCGAGGACGCGCGGAGCCCCCG------CCGTGGCCGGGACCGCGGGCTGGAACGCCCCC
intest4         GCGAGGACGCGCGGAGCCCCCG------CCGTGGCCGGGACCGCGGGCTGGAACGCCCCC
adreae1         GCGAGGCCGGGCGACCCGCCAAGCCGGCCCGTGGTTGGGATCGCGGGCTGGAACGCCCCC
ibis            GCGAGGCCGGCCGACCCGCCAAACCGGCCCGTGGTTGGGATCGCGGGCTGGAACGCCCCC
muris2          GTTGTGAAGCTTGATGAGATCAACCCCCACGTGGTTGGGATCGTGGACTGGAACGTCCTC
vole            GCGAGGACGCGCGGAGCCCCCG------CCGTGGCCGGGACCGCGGGCTGGAACGCCCCC
hamster1        CTTAAGGGACGTGGGTGGTGAACCCGTCACNTGGTTGGGATCGTGGGCTGGAAGGGCCAC
                     *     *                * ***  **** ** ** ****** * ** *

intest_1        GCGCACCAGG-AATGTCTTGTAGGCGCGCGCCCCCACCGCGCGCCGGACGCGTCCCTGCC
intest2         GCGCACCAGG-AATGTCTTGTAGGCGCGCGCCCCCACCGCGCGCCGGACGCGTCCCTGCC
intest4         GCGCACCAGG-AATGTCTTGTAGGCGCCCGCCCCCCACCGCGCGCCGGACGCGTCCCTGCC
adreae1         GTGAACCCGG-AATATCTCGTAGGCGCGTGTCCCCAACGCGCGCCGGATGCGTCCCTGCC
ibis            GTGAACCCGG-AATATCTAGTAGGCGCGTGTCCCCAACGCGCGCCGGATACGTCCCTGCC
muris2          GTGAACCTGG-AATGTCTAGTAGGCGTAGGTCATCAATCTACGCCGGATACGTCCCTGCC
vole            GCGCACCAGG-AATGTCTTGTAGGCGCGCGCCCCCACCGCGCGCCGGACGCGTCCCTGCC
hamster1        GTGAACCTGGTAATGTCTAGTAGGCGTAGGTCATCAATCTACGCCGGATACGTCCCTGCC
                * * *** ** *** *** *******   * *  **     *******  **********

intest_1        CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGCGCCCCGGACGC
intest2         CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGCGCCCCGGACGC
intest4         CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGCGGCGAGCGCCCCGGACGC
adreae1         CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCCGCGGCGAGTCCCCGGGAGCC
ibis            CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCCGCGGCGAGTCCCCGGGAGCC
muris2          CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGTCTTCTGGCGAGCTCCTGGGAGGG
vole            CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGCGCGGGCGAGCGCCCCGGACGC
hamster1        CCTTGTACACACCGCCCGTCGCTCCTACCGACTGGGTGTCATGGCGAGGCCCTTGAGG--
                ************************************     ******  **  *

intest_1        -----GCGAAGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTAT-
intest2         -----GCGAAGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTAT-
intest4         -----GCGAAGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTATT
adreae1         CCCG-GTGAACGGCGACGAGCCCTGGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTAT-
ibis            CCCG-GCGAACGGCGACGAGCCCTGGCGCTTGGAGGAAGGAGAAGTCGTAACAAGGTAT-
muris2          ATGAACCGAACAGGGACGAACCGCGAGGCTTGGAGGAAGGAGAAGTCGTAACAAGGTAT-
vole            -----GCGAAGGGCCGCGAGCCCCCGCGCCTGGAGGAAGGAGAAGTCGTAACAAGGTAT-
hamster1        ---------ATAGGGACGAGCCATGGGACTTGAAGGAAGGAGAAGTCGTAACAAGGTAT-
                         *  *   *** **      * ** ****************************
```

```
intest_1        CCGTAGGTGAACCTGCGGATGGATCCCTCGCGCGCGGCGTGTGTCGCCCCCGCGGCCCGG
intest2         CCGTAGGTGAACCTGCGGATGGATCCCTCGCGCGCGCGGCGTG-CGTCCCCGCGGCCCGG
intest4         CCGTAGGTGAACCTGCGGATGGATCCCTCGCGCGCCCCGCGCGTCGCCCCCGCGGCCCGG
adreael         CCGTAGGTGAACCTGCGGATGGATCCCTAGGCGGACCGGTCCGGTCCCCGGGCGGCGTAT
ibis             CCGTAGGTGAACCTG----------------------------------------------
muris2          CCGTAGGTGAACCTGCGGATGGATCCATCGAGAGGGAA-------GGTACGAGGATGAAG
vole            CCGTAGGTGA--------------------------------------------------
hamster1        CCGTAGGTGAACCTGCGGATGGATCCAT----------------GGCCCAAAGGATGAG
                **********

intest_1        TCG--GCACGCGAGCCCCGCGCCGGCGGATGCCTCGGCCCGGGCGGCGACGAAGAGCGCG
intest2         TCG--GCACGCGAGCCCCGCGCCGGCGGATGCCTCGGCCCGGGCGGCGACGAAGAGCGCG
intest4         TCG--GCCCCCGAACGCCCCGCCGGCGGATGCCTCGGCCCGGGCGGCGACGAAGAGCGCG
adreael         CCCCTGGCATGCAGACCCAGACCGGCGGATGCCTGGGCTCGGGCCTCGAGGAAGGACGCG
ibis             ------------------------------------------------------------
muris2          GCA---ATGAGATGACGCGACCCGGTGGATGCCTTGGCTCGGGGGACGATGAAGGACGTG
vole            ------------------------------------------------------------
hamster1        ATG---AGAAGAGGAC-CAACCCGGTGGATGCCTTGGCTCGGGGGACGATGAAAAACGTG

intest_1        GCGGAGCGCGAGACGCGGTGCGGACC-CGCCCGCCCCGAGAAGCACCGACCCTCGAACGC
intest2         GCGGAGCGCGAGACGCGGTGCGGACC-CGCCCGCCCCGAGAAGCACCGGCCCTCGAACGC
intest4         GCGGAGCGCGAGACGCGGTGCGGACC-CGCCCGCCCCGAGAAGCACCGACCCTCGAACGC
adreael         GCGGACAGCGAGATGCGGTGCAGCCC-GGCACGAAGCACCGGTTTCCGAACGCTCGGCCT
ibis             ------------------------------------------------------------
muris2          GCTGACGACGATACTCGATGTGGTCCAAGCACGTGACATCGATCTTCGAATGGATCATCG
vole            ------------------------------------------------------------
hamster1        GTGGACGACAAGATACGATTTGGAT--GGCATGTGACATCCACCTTCCAA-AGCTGTCCA

intest_1        AGCGCGCCCCGGCGCCGCCGCCTCGGCGCCCGCCGCGTGCCGC-----------------
intest2         AGCGCGCCCCGGCGCCGCCGCCTCGGCGCCCGCCGCGTGCCGC-----------------
intest4         AGCGCGCCCCGGCGCCGCCGCCTCGGCGCCCGCCGCGTGCCGCGCGCCGCGCCGCAGAGA
adreael         CCCCCGGGAGACCGGCCGCTCGGCGGGGGCAGCCTCCCGCGGGCCCCGCGGCGAGCCCAC
ibis             ------------------------------------------------------------
muris2          GGGTGGTGGGGTGATCTACGAACTGGGTTGTTCGAGGGGTTGAACGATGGGGAATGAGAT
vole            ------------------------------------------------------------
hamster1        ATCCCGTGGGGGGA--CACAACCTA--CCAACCAAGGGG----------------------

intest_1        ------------------------------------
intest2         ------------------------------------
intest4         GCGCCCCGGGGCGGTCCCGCCGGGCTGCGCGGCCCGAG
adreael         GCACGCGCTCTGCCCGGAGCGACTCC------------
ibis             ------------------------------------
muris2          GAGGATCCCCCCCCACTCCGATGAAGAT----------
vole            ------------------------------------
hamster1        ------------------------------------
```

Fig. 8 Splitsgraph showing relationships between different isolates of *Giardia* as inferred from 204 homologous nucleotide positions. Insertions and deletions were excluded for the purpose of splitsgraph construction.



Giardia intestinalis isolates - intest1, intest2, intest4
Giardia muris isolate - muris2
Giardia ardeae isolate - ardeae1
Giardia isolate from ibis' - ibis
Giardia isolate from voles - vole
Giardia isolate from hamster - hamster1

Chapter 4. Discussion

4.1 Generation of *in vitro Giardia intestinalis* cysts from trophozoite culture

Using this *in vitro* method of generating *Giardia intestinalis* cysts, the cyst yield was quite high with a yield of 3.5 X 10^5 cysts/ml on the fourth day of incubation, from an original trophozoite concentration of 7.2 X 10^5 organisms/ml. However, viable cysts were not produced. Viability as measured by excystation and by fluorogenic dye staining on five batches of *in vitro* cysts of different strains originating from humans, sheep and beavers resulted in 100% non-viable results. This may be due to the strains of *Giardia intestinalis* used. It is possible that viable cysts may have been generated using different strains. The production of viable *in vitro* cysts has been documented (Boucher and Gillin, 1990; Schupp *et al.*, 1988b). In a population of cysts from a natural environment, for instance calf faeces, tests showed that the viability of *Giardia* cysts in nature is usually between 80% and 95%.

4.2 Use of DAPI and PI to measure the viability of cysts

The standard method of measuring viability at present is by *in vitro* excystation. This process mimics the conditions encountered by the cyst on its passage through the host. The problem with using excystation as a measure of the viability of *Giardia* cysts is that cysts of different strains appear to have slightly differing requirements in order for *in vitro* excystation to take place. Therefore the reliability and repeatability is not high. The excystation protocol described for the viability studies was successful in excysting a human *Giardia intestinalis* sample and another *G. intestinalis* sample isolated from calf faeces. However, for subsequent batches of *G. intestinalis* cysts isolated from calf faeces, successive attempts resulted in excystation percentages ranging from 0% to 10%. The cysts themselves were fresh and otherwise displayed the normal morphology of viable cysts under phase contrast microscopy - smooth and hyaline. Fresh cysts from

faecal sources typically had viability rates of 80% to 95%. It may be that cysts from different sources in the natural environment are comprised of different strains of *Giardia intestinalis*. In addition, it appears cysts may require a 'ripening' time - excystation protocols which fail to excyst fresh cysts have been found to be more effective once the cysts are about a week old (Bingham and Meyer, 1979; Schaefer *et al.*, 1984). These particular cysts excysted at very low levels at all times between the time of collection and six weeks subsequently.

It is also possible that observing the mechanism of cyst excystment may be inadequate in assessing the viability of the trophozoites released. The standard method for calculating viability using excystation (Sauch, 1988) counts partially excysted trophozoites as excysted and therefore viable. Viable trophozoites have been observed to escape from the cysts by vigorous use of their flagellae (Schaefer *et al.*, 1984). The initial cracking open of cysts may be merely a mechanical reaction in response to an acidic environment, bearing little relation to the state of viability of the cysts. Many of the partially excysted trophozoites seem to never completely excyst. It is possible then that counting partially excysted trophozoites may overestimate the number of infective cysts present (DeRegnier *et al.*, 1989).

The excystation process itself involves a number of steps and chemicals so that many combinations of the conditions are possible. Schaefer *et al.* (1984) found that the method of excystation used by Bingham and Meyer (1979) was unsuccessful in excysting their specific cyst population. If a particular ***in vitro*** excystation protocol is found not to work on a type of cyst, a large sample of the cysts are needed to test for the optimal conditions for excystation. However, it would be difficult to obtain adequate numbers of cysts from environmental water samples. In addition, approximately 10^4 to 10^5 cysts are required per excystation, whereas environmental water samples typically yield 1 to 100 cysts for tests filtering 380 L of water. Even where a successful method is found, it is difficult to measure whether this is optimal, as is accurate replication.

For the viability experiment at 15°C in milli-Q water and sea water, excystation was used to measure the viability of *G. intestinalis* cysts isolated from human faeces. Although the same protocol was used for measurement at each point, the graph of

viability does not show a downward progression during some periods. In Fig. 1, the proportion of viable cysts was recorded as rising between day 2 and day 5 in both the milli-Q water and sea water. The proportion of viable cysts in milli-Q water fell from 49% to 31 % over 2 days, but took a further 9 days to fall 10% to 21%. The results for the sea water also looked odd with viability measurements rising between day 5 and day 9. Experiments using fluorogenic dyes to measure viability showed a less erratic downward progression in viability.

Fluorogenic dye staining is another method of determining the viability of cysts. One recognised method (Jones and Sneft, 1985) uses a combination of two fluorogenic dyes, fluorescein diacetate (FDA) and propidium iodide (PI). FDA lights up viable cysts apple-green using a Reichart-Jung epifluorescent microscope under a blue filter with an excitation wavelength of 450-490 nm. It diffuses into cysts, and non-specific esterases release free fluorescein which diffuses through intact lipid bilayers. In the determination of cell viability, an intact lipid bilayer slows the leakage of FDA from within intact cells, while damaged cells cannot retain or accumulate the fluorescein. In *Giardia* cysts, the fluorescein accumulates within the trophozoites and in the space between the trophozoite membrane and the cyst wall so the cyst wall may be an additional barrier to the release of fluorescein. Propidium iodide (PI) cannot traverse intact cell membrane, so penetrates only damaged cyst walls and highlights the nuclear material. It therefore lights up only dead cysts (Smith and Smith, 1989). These appear red under a green filter with an excitation wavelength of 515-560 nm. Samples of cysts are stained with these dyes simultaneously so that it is possible to view the same cyst under the different filters to assess their viability.

However, when this method using FDA and PI was tried, it was found that no cysts were stained with FDA in samples of fresh cysts, for which viability rates of 80% to 95% would be expected. 'Black cyst' shapes (Schupp *et al.*, 1987a) were often observed under the blue filter, which were also not stained with PI, indicating where possible viable cysts were. For the cyst specimens used, FDA was ineffective in measuring viability. The cysts used were *G. intestinalis* cysts from fresh calf faeces. An additional problem with this method is that the cysts stained with FDA are viewed under the blue filter which is also used for the detection of cysts stained with fluorescein-labelled

monoclonal antibody, so it is not possible to concurrently stain a sample with a monoclonal antibody kit to first locate cysts and from there to check their viability.

An alternative fluorogenic dye to FDA is 4',6-diamidino-2-phenylindole (DAPI) which also highlights nucleic acids. It is an AT-selective DNA stain, and when binding to DNA occurs there is an approximate 20-fold enhancement in fluorescence. Both viable and non-viable cysts can be stained with DAPI and are visible under a UV filter at an excitation wavelength of 340-380 nm. This means it is possible to carry out fluorogenic dye-staining simultaneously with DAPI, PI and fluorescein-labelled monoclonal antibody so that cysts can be located and their viability assessed. A method using this combination of fluorogenic dyes has been developed to assess the viability of *Cryptosporidium* oocysts (Campbell *et al.*, 1992). The concentration of PI used by Schupp and Erlandsen (1987a) for assessing *Giardia* viability was found to be barely visible for the cysts used. The PI concentration used by Campbell *et al.* (1992) for *Cryptosporidium* was therefore trialled and found to be more useful in distinguishing between cysts and debris.

For viability stains of *Cryptosporidium* oocysts with DAPI, the nuclei of the sporozoites within the oocysts are visible for viable oocysts. It was expected that *Giardia* would be similar in that the nuclei of trophozoites would be visible in viable cysts. A staining time of ten minutes at 37°C seemed sufficient to stain the cysts with both DAPI and PI. Any cysts which took up the PI stain (PI+ cysts) (Plate 2, B) were classed as non-viable. Cysts which were PI+ were invariably stained with DAPI (DAPI+) (Plate 2, A) as well. It was found that the trophozoites' nuclei of a large proportion of cysts did not stain with PI, indicating that they were viable, but neither did they stain with DAPI (DAPI-/PI- cysts) (Plate 1, A). The outlines of the cysts were clearly visible under UV light which indicated that the cysts were to a certain extent stained with DAPI.

In the study by Robertson *et al.* (1992) on the survival of *Cryptosporidium* oocysts, it was found that different isolates of oocysts varied in their permeability to the DAPI dye, and that a certain proportion included neither DAPI nor PI (DAPI-/PI-). These oocysts were viable. The DAPI- oocysts were transformed to DAPI+ cysts over time.

When the time for which the cysts were incubated with the stains was extended, it was found that increasing the stain times had an effect on the permeability of the *Giardia intestinalis* cysts to the fluorogenic vital dyes. The number of nonviable (PI+) cysts rose during a 2 hour stain time and the number of DAPI+/PI- cysts, those whose nuclei were stained with DAPI, also increased over a 60 minute stain time. It seems that the DAPI-/PI- cysts are viable cysts which require a longer stain time in order for the nuclei to become stained. There also appears to be good correlation between viability measurements using *in vitro* excystation and fluorogenic dye staining.

During the viability experiments, it was also found that as time went on and the number of viable cysts decreased, the number of DAPI+/PI- cysts increased. For a fresh sample of cysts with viability readings of 85%-90%, typically 5% of the cysts were DAPI+/PI-, the rest of the viable cysts being DAPI-/PI- cysts. As the number of viable cysts decreased, the proportion of these displaying DAPI+/PI- staining behaviour increased. By the time viability levels reached 10%, all viable cysts showed DAPI+/PI- staining behaviour.

Cysts which had been stored in sea water presented no staining difficulties. Viable and non-viable cyst-types were easily distinguished using the DAPI and PI stains. It had been expected that sea water would cause changes in the permeability of the cyst wall, preventing the uptake of the vital dyes. Using the fluorescein-labelled monoclonal antibody stain, some cysts were difficult to detect. Previous work (Donaghy, 1993) indicated that cysts stored in sea water displayed erratic staining behaviour when the FDA vital dye was used to assess viability, with viability readings using FDA rising to 100% after 1-2 days (Donaghy, 1993). There is a suggestion that sea water may affect the permeability of the cyst to FDA, but the DAPI stain did not seem to present any such difficulties.

4.3 Viability of cysts over time

In this study, the viability of *Giardia intestinalis* cysts under different temperature conditions and in sea and deionised water was assessed using the fluorogenic dyes DAPI and PI.

The *Giardia intestinalis* cysts used in all the viability experiments were isolated from bovine faeces. The cysts which had been stored at 4°C in purified milli-Q water remained viable for over 62 days (Fig. 2). This period of survival was extremely long. The viability of the cysts remained high for much of this time. On the 48th day, the viability level was still 36%. After this, the level of viable cysts fell sharply, from 36% to 2% in two weeks.

The cysts stored at 10°C remained viable for a similar period to those stored at 4°C, but at any time leading up to the sixtieth day, the number of viable cysts was consistently higher for those stored at 4°C (Fig. 2).

At a storage temperature of 15°C (Fig. 2), the survival time of the cysts shortened considerably, from 62 days at 10°C to 17 days. This was the greatest reduction in survival time over any 5°C temperature increase. Low temperatures appear to be important for the long-term survival of cysts in the environment.

At a temperature of 20°C (Fig. 2), the survival time was shortened again, to 7 days. The cysts stored at 25°C (Fig.2) were inactivated in a period of 4 days. Considering that 25°C is a high temperature for environmental water, the fact that cysts can remain viable for up to 4 days after exposure to this temperature has serious implications for recreational water areas. Environmental waters in New Zealand probably cover the whole range of temperatures from 4°C to 25°C. When cysts are found in water samples, the possibility cannot be ruled out that these are viable. The temperature at which cysts are stored has a considerable impact on their viability. When stored at 15°C, the period

for which they remain viable dropped to 17 days, less than one-third of the time they remained viable at 4°C. The results obtained here are in agreement with the findings of DeRegnier *et al.* (1989) that cysts exposed to temperatures below 10°C remained viable for 56-84 days, and that cysts at temperatures higher than 10°C had reduced viability. Bingham and Meyer (1979) found that cysts remained viable for up to 77 days when stored at 8°C. There is little other information available, but these studies agree with the results found here.

The sea water had an immediately lethal effect on the *Giardia intestinalis* cysts (Fig. 3). Samples of cysts in sea water were set up concurrently with the cysts stored in milli-Q water at the various temperatures monitored. When viability measurements were taken after one day, all the samples stored in sea water were 100% non-viable. An environment of 4°C was then chosen for the sea water experiment as the cysts survived for the longest period in milli-Q water at this temperature. It was found that in the sea water environment, cyst viability dropped to zero in less than an hour. At time zero, where cysts were immersed in sea water and immediately washed in milli-Q water, the cyst viability dropped from 54% to 10.6%. Combined with the immediate diluting effect of the sea water, *Giardia* cysts in sea water would seem to present little risk of infection.

4.4 Analysis of Hamster *Giardia* rDNA

The *Giardia* cysts isolated from the faeces of the infected hamsters were stained using an MGI-1 Multi-Glo Indirect kit, developed to distinguish between *Giardia intestinalis* and *Giardia muris* (McLenachan *et al.*, in preparation). Using an epifluorescent microscope, *G. intestinalis* cysts are labelled with fluorescein and appear green while *G. muris* cysts are indirectly stained with R-phycoerythrin and appear orange-yellow, under a blue filter. The hamster *Giardia* cysts appeared yellow, with a green tinge. This indicates that they stain weakly with fluorescein and strongly with R-phycoerythrin. The staining behaviour of the cysts was very similar to that of *G. muris*, but they were distinguishable from the *G. muris* positive control cysts.

A comparison of the morphology of *Giardia intestinalis* and the *Giardia* from the hamsters under scanning electron microscopy (SEM) is shown in Plates 3 and 4. These show little difference between the two types from a dorsal viewpoint. They have a similar shape, and are convex with a roughened dorsal surface (Plate 3). From the SEM photo of the dorsal surface of the *Giardia* isolated from hamsters, it is possible to see the presence of two caudal flagellae (CF), two posterolateral flagellae (PLF) and two anterolateral flagellae (ALF) (Plate 3, B).

The difference between the two types of trophozoite can be seen on the ventral side (Plate 4). For the *G. intestinalis* trophozoite (Plate 4, A), the ventro-lateral flange (VLF) forms a continuous border around the adhesive disc, so that where the two ventral flagellae (VF) emerge, the flange appears as a raised flap. In the hamster *Giardia* trophozoite (Plate 4, B), the ventral adhesive disc has a deep notch at the point where the ventral flagellae emerge. The ventro-lateral flange does not continue around the notch, giving it a cut-off appearance. A similar notch can be seen in *Giardia muris* trophozoites. There is also a notch in the same position on the ventral side of *Giardia ardeae* trophozoites (Campbell *et al.*, 1990). *G. ardeae* trophozoites though have a single caudal flagellum, whereas both *G. muris* and the *Giardia* isolated from the hamsters have two caudal flagellae. As hamsters are rodents, it would seem more likely that the trophozoites resemble *G. muris* from rodents most closely. From electron microscope photographs, it seems that in morphology the *Giardia* isolated from hamsters is closest in appearance to *G. muris*.

The yield of DNA from hamster *Giardia* cysts treated with the 10% Chelex-100 resin was sufficient for use in PCR analysis. Approximately 1 X 10^3 cysts were used in the DNA extraction. This method is particularly useful for analysing environmental samples of *Giardia* as it is difficult to extract DNA from the cyst form of *Giardia*, and difficult also to excyst and cultivate trophozoites for DNA extraction from cysts isolated from water samples. The cyst wall is very resistant to environmental conditions, making it hard to crack open. The harsh boiling treatment the cysts undergo with this method appears to be abrasive enough to achieve this.

In different organisms the rRNA genes are conserved in function, organisation and sequence. They vary sufficiently to be a useful basis for characterisation by comparing sequences for similarities and differences. The rDNA of *Giardia* consists of a external non-transcribed spacer (ENTS), the small-subunit (SSU) rRNA gene (16S rRNA), an internal transcribed spacer (ITS), the 5.8S rRNA gene, another ITS, and the large sub-unit (LSU) rRNA gene (23S) (van Keulen *et al.*, 1992). This is tandemly repeated.

The PCR primers which were used to characterise the rDNA were developed from the total nucleotide sequence for the rDNA for *Giardia intestinalis* of Healy *et al.* (1990). The position of these primer pairs along the rDNA are shown in Fig. 4. These primers were used for the PCR analysis of the hamster *Giardia* rDNA. The rDNA of *G. muris* and *G. intestinalis* were analysed concurrently for comparison and as a positive control. Hamster *Giardia* DNA extracted from trophozoites in hamster gut as well as DNA from chelexed cysts isolated from faeces was used. The *G. intestinalis* DNA used was from an Australian isolate, Bris-HEPU, and the *G. muris* DNA was from a USA isolate, Seattle3.

The results of PCR, using the various primer pairs are shown in Fig. 4 and Table 4. Of the primer pairs used, only four successfully produced a sample for all three *Giardia* types: Uno-1 and Uno-2, Joli-1 and Joli-2, Ace-1 and Koni-2 and Thor-1 and Thor-2. Various primer combinations produced samples which covered the section of *G. intestinalis* rDNA from 16SR-1 to Thor-2. No PCR samples were produced for the section of rDNA from 16SR-1 to Uno-1 for *G. muris* or the hamster *Giardia*. Published rDNA sequences for *G. muris* showed that this region of the rDNA varies markedly from *G. intestinalis* (van Keulen *et al.*, 1992; Healy *et al.*, 1990) It seems likely that the primers were too dissimilar to anneal to this section of the rDNA of *G. muris* and the hamster *Giardia*. Using combinations of primer pairs, PCR samples were obtained for the region between Uno-1 and Thor-2 for the *G. muris* rDNA. Three sections of the hamster *Giardia* rDNA could not be amplified by PCR – between 16SR1 and 16SR2, between Joli-2 and Ace-1 and between Koni-2 and Thor-1.

From comparison of published sequences of the rDNA for *G. intestinalis, G. muris* and *G. ardeae* (van Keulen *et al.*, 1992) it appears that the sequences of the 16S, 5.8S and

23S regions were for the most part quite conserved. Any sequence variation between species tend to occur in the ENTS region, the ITS region and on the ends of the 16S, 5.8S and 23S regions. In order to investigate the relationship between *Giardia* from hamster, *G. intestinalis* and *G. muris*, it was decided to sequence one of the fragments in common. The Joli-1/Joli-2 fragment was chosen as a useful section to sequence for characterising features since at 616 bp in length it was not overly long, yet it covered the region of the two internal transcribed spacer regions, as well as the 5.8S region. Moreover, it was a region readily amplified by PCR for all three species (Fig. 5). Both the *G. muris* and *Giardia* from hamster were successfully sequenced on a sequencing gel, and the hamster Joli fragment was also sequenced using an automatic sequencer (Fig. 6).

Several other sequences were added from the database in order to give a clearer idea of how closely related the hamster *Giardia* isolate was to *Giardia muris*. These included 3 additional defined strains of *G. intestinalis*, *Giardia microti* (a strain of *Giardia* isolated from voles), and a strain of *Giardia* isolated from ibis. The Genebank accession numbers are shown in Appendix A.

The sequences were aligned using CLUSTAL (Fig. 7). A splitsgraph (Bandelt and Dress, 1992) (Fig. 8) was used to represent the relationships between the isolates as inferred from 204 homologous nucleotide positions. This included four strains of *G. intestinalis*, *G. microti* from voles, *G. muris*, *G. ardeae*, *Giardia* from ibis and the hamster *Giardia*. Insertions and deletions were excluded for the purpose of the splitsgraph construction.

On the splitsgraph there are three distinct groupings of the various isolates. The *G. microti*, perhaps surprisingly, groups with the *G. intestinalis* strains indicating that it is a strain of *G. intestinalis*. The *Giardia* from ibis groups with *G. ardeae*, which is not unexpected as both are found in birds. The *G. muris* and *Giardia* from hamsters group together, which again might be expected as both are from rodents. The group consisting of the *G. muris* and *Giardia* from hamsters is separate from the other two groups. However, the differenes between the *G. muris* and *Giardia* from hamsters are greater than between the strains of *G. intestinalis*. Between defined species of *Giardia* – *G.*

intestinalis, *G. muris* and *G. ardeae* - there were about 30 base differences in the 204 base pair region. Between strains of one species, *G. intestinalis*, there were 1 or 2 bp differences. However, between the hamster *Giardia* and the *G. muris* species there are 10 bp differences. This would suggest that the sequence of the hamster *Giardia* is too similar for it to be considered a separate species, yet too divergent to be considered merely one strain of *G. muris*. The differences may be sufficient to warrant the hamster *Giardia* being named a subspecies of *G. muris*.

Using PCR directed at the rDNA of *Giardia* appears to be a convenient and effective method of analysing the genetic variations in *Giardia* isolates. The 'Joli' fragment used in the directed PCR method spans the small 5.8 S unit, which tends to be a conserved region between species, and the more variable spacer regions between the 16 S and the 5.8 S, and between the 5.8 S and the 23 S region. Examination of this small region of the rDNA for defined nucleotide changes is a highly convenient method of differentiating between strains of *Giardia*. Cyst concentrations in environmental water samples tend to be low, so finding the source of waterborne outbreaks of *Giardia* is very difficult. Isaac-Renton *et al.* (1993) traced the source of one outbreak to a diseased beaver by trapping and extracting the gut contents of animals, a labour-intensive job. Reports indicate that the DNA from one organism is sufficient for PCR analysis.

Appendices

Appendix A: Data

Data for Fig. 1 - Viability of *G. intestinalis* cysts isolated from human faeces at 15C in milli-Q and sea water as measured by excystation

Days	Milli-Q	Sea
0	**49.0%**	**39.8%**
1	40.0%	33.9%
2	**30.9%**	**27.9%**
3	31.8%	23.8%
4	32.6%	19.6%
5	**33.5%**	**15.5%**
6	32.5%	15.8%
7	31.5%	16.0%
8	30.4%	16.3%
9	**29.4%**	**16.5%**
10	25.3%	16.0%
11	**21.2%**	**15.5%**
12	21.2%	12.9%
13	**21.2%**	**10.3%**
14	20.4%	9.2%
15	19.7%	8.0%
16	18.9%	6.9%
17	18.2%	5.7%
18	17.4%	4.6%
19	16.7%	3.4%
20	15.9%	2.3%
21	14.8%	1.1%
22	**14.4%**	**0.0%**
23	11.0%	
24	7.6%	
25	**4.2%**	
26	3.2%	
27	2.1%	
28	0.8%	
29	**0.0%**	

The highlighted figures were the measured percentages, while the remainder were calculated for the purposes of drawing a line graph

Data for Fig. 2 - Viability of *G. intestinalis* cysts from calf faeces at 4C, 10C, 15C, 20C and 25C in milli-Q water as measured by DAPI and PI

Days	4C	10C	15C	20C	25C
0	**85.0%**	**85.0%**	**79.5%**	**49.0%**	**85.7%**
1	83.7%	80.2%	75.4%	46.5%	**62.0%**
2	82.3%	75.3%	71.3%	**43.5%**	**36.0%**
3	81.0%	**70.5%**	67.1%	33.6%	**7.5%**
4	79.7%	65.7%	63.0%	23.3%	**0.0%**
5	78.4%	60.8%	54.2%	**13.0%**	
6	**77.0%**	**56.0%**	**45.4%**	7.0%	
7	76.0%	56.1%	36.6%	**0.5%**	
8	75.0%	56.3%	27.8%		
9	74.0%	56.4%	19.0%		
10	73.0%	**56.6%**	16.0%		
11	72.0%	56.7%	13.0%		
12	71.0%	56.9%	11.5%		
13	**70.0%**	**57.0%**	**10.0%**		
14	68.3%	54.6%	8.5%		
15	66.6%	52.1%	5.5%		
16	64.9%	49.7%	2.5%		
17	63.1%	47.3%	**0**		
18	61.4%	44.9%			
19	59.7%	42.4%			
20	**58.0%**	**40.0%**			
21	57.6%	39.4%			
22	57.1%	38.7%			
23	56.7%	38.1%			
24	56.0%	37.4%			
25	55.9%	36.8%			
26	55.4%	36.1%			
27	**55.0%**	**35.5%**			
28	54.6%	33.1%			
29	54.1%	30.8%			
30	53.7%	28.4%			
31	53.3%	26.1%			
32	52.9%	23.7%			
33	52.4%	21.4%			
34	**52.0%**	**19.0%**			
35	50.9%	18.3%			
36	49.7%	17.6%			
37	48.6%	16.9%			
38	47.4%	16.1%			
39	46.3%	15.4%			
40	45.1%	14.7%			
41	44.0%	**14.0%**			
42	42.9%	13.3%			
43	41.7%	12.6%			
44	40.6%	11.9%			
45	39.4%	11.1%			
46	38.3%	10.4%			
47	37.1%	9.7%			
48	**36.0%**	**9.0%**			
49	30.8%	7.8%			
50	25.6%	6.6%			

Data for Fig. 2 continued.

Day	4C	10C
51	20.4%	5.4%
52	15.2%	4.2%
53	**10.0%**	**3.0%**
54	9.1%	2.7%
55	8.2%	2.3%
56	7.3%	2.0%
57	6.4%	1.7%
58	5.6%	1.3%
59	4.7%	1.0%
60	3.8%	0.7%
61	2.9%	0.3%
62	**2.0%**	**0.0%**

The highlighted figures were the measured percentages, while the remainder were calculated for the purposes of drawing a line graph.

Data for Fig. 3 - Viability of *G. intestinalis* cysts isolated from calf faeces at 4C in sea water, as measured by DAPI and PI

Minutes	**Viable cysts**
0	**10.6%**
15	**7.5%**
30	**2.0%**
45	**0.0%**

Sequence data for *Giardia* from hamsters (from sequencing gel)

Joli-1 (long) 30/7/96
TTG TAC CAC ACC CCT CGT CCT ACC GAC CTC
GGG CTC CTC ATG GCG ACG CGC CCT TGA GGA
TCA GGG ACG AGC CAT CGG ACT TAA GGA AGG
AGA AGT CGT AAC AAG GTA TCC TAG TAA cCT
gGA TGA TCA TGC AAG ATG AGA TGG AAG AGG
ACA CGT GAT GCT GCT CgG ACG ATG AGA CGT
GTG ACG ACG AGA ATA CGA TTG ATG CAT TAC
ATC ACT CAG CTT CAT CTG gCA aCT

Joli-1 (long) 8/8/96
GGA ATT GCT AGT AGG CGT AGG TCA TCA ATC
TAC CGC GGA TAC GTC CtC TAG CCa CCT TTG
TTG ACA CAC CGc CCT GCc GRC CTA CCG ACT
GGT GTG CAT GCG GAG GCC TTA GGG ATA GGG
ACG ACG ATA GGA CTT AGA GAA GGA TAG AaT
CGT GAA CAT GAT CTA TGA CTC GAT GAT CAT
CAA AGT AGA GTA GAG AGG ACA C

Joli-1 (short) 8/8/96
GTt GAC Gtt ATA GGT Gta CtG TGG GTG TGT
ata tCC Cgt TCT ACG TGG TTG TGT GAT CGT
GGG CTG Gta atG GtG CAC GTt GAT ACt CTG
Gta aTG TCT AGT AGG CGR AGG TCA TCA aTC
TAC GCG GRA CGR CcT Gcc TTG RAC ACA CGC
cTG CcG TCT ACG ACT GgT TGC ATG GAG CTG
AGT AGR CAG AGC TAG ACT GAG cA

Joli-2 (long) complement reverse 8/8/96
CAT GCA GcC TAG ATA GAC ACA TGA CTA GAG
AAG TCG TAC TAG TAT CGT AGR GAA CTC GAT
GAT CCA TGg CCC AAA GAT GAG ATG AGA AGA
GgA CCA ACC CGG TGG ATC CTT GGC TCG GGG
GAC GAT GAA GAA CGT GGT GGA CGA CGA GAT
ACG ATG TGG ATG GCA T

Joli-2 (short) complement reverse 8/8/96
ATC GAA GAT AAT AAA GAC ACA CGT GAT cGC
CaT aGC TCG GAC GAT GAA GAA CGT GGT GAC
GAC GAG ATA CGA TGT GGA TGg CAT GTG ACA
TCG AcC TTC GAA GCT GTC GAT TCG GTG ggg
gAa tCA CAA CCT ACG ACC AA

Sequence data for *Giardia* from hamsters, from Joli-1 end (from automatic sequencer)

TnA GGA TCT GGG TTG CGG TTT TAA TAC GGG
GTG ACG GGG GAT GCT CGA CGT GCA nTA AGG
GnC GTG GGT GGT GAA CCC GTC CAn TGG TTG
GGn TCG TGG GCT GGA AGG GCC ACG TGA ACC
TGG TAA TGT CTA nTA nGC GTA GGT CAT CAA
TCT ACG CCG GAT ACG TCC CTG CCC CTT GTA
CAC ACC GCC CGT CGC TCC TAC CGA CTG GGT
GTC ATG GCG AGG CCC TTG AGG ATA GGG ACG
nnC CAT GGG ACT TnA AGG AAG GAG AAG TCG
TAA CAA GGT ATC CGT AnG TGA ACC TGC GGA
TGG ATC CAT GGC CCA AAG GAT GAG ATG AGA
AGA GGA CCA NCC CGG TGG ATG CCT TGG CTC
GGG GGA CGA TGA AAA ACG TGG TGG ACG ACA
ANA TAC GAT TTG GAT GGC ATG TnA CAT CCA
CCT TCC AAA GCT GTC CAA TCC CGT GGG GGG
ACA CAA CCT ACC AAC CAA GGG GGT TTC AAA
GGA AAA TCT CCG GAA CCn AAC TCC TGA ACC
CCC CCC CnA ATC CAC CTT GAT ACA TGT CTn

Sequence data for *Giardia* from hamsters, from Joli-2 end (from automatic sequencer)

CTA GGA nAT ACG TnT AGG GGn TAA TTn nnG
AAA AnG TCT GGn CCn CAG ATC Tnn ACT CGA
CCC CCT TGG TCG TAG GTT GTG TCC CCC CAC
CGA ATC GAC AGC CTT CGA AGG TCn ATG TCA
CAT GCC ATC CAC ATC GTA TCT CGT CGT CCA
CCA CGT TCT TCA TCG TCC CCC GAG CCA AGG
CAT CCA CCG GGT TGG TCC TCT TCT CAT CTC
ATC CTT TGG GCC ATG GAT CCA TCC GCA GGT
TCA CCT ACG GAT ACC TTG TTA CnA CTT CTC
CTT CCT TCA AnT CCC ATG GCT CGT CCC TAT
CCT CAA GGG CCT CGT CAT GAC ACC CAG TCG
GTA GGA nCG ACG GGC GGT GTG TAC AAG GGG
CAG GGA CnT ATC CGG CGT AnA TTG ATG ACC
TAC GCC TAC TAA ACT TTC CAG GTT CAC nTG
GCC CTT CCA GCC CAC AAT CCC AAC CAC GTT
ACG GTT CCC AAC CCA CnT CCC TTA AGT TCA
ATT CGG GCA TTC CTC nTC ACA nTG TGC ACC
CAG GGC GTC ATT nGG CAT AAG A

Accession numbers

Giardia intestinalis 1 – GenBank - UO9491

Giardia intestinalis 2 – GenBank – U09492

Giardia intestinalis 4 – EMBL accession no. X52949

Giardia ardeae – EMBL accession no. X58290

Giardia muris – EMBL accession no. X65063

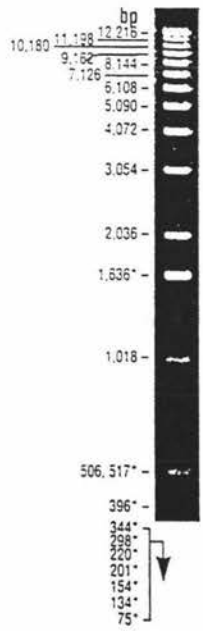
Giardia microti (vole) – AF006676

Giardia from ibis – GenBank U20351

Appendix B

Reagents

The DNA ladder used to size the PCR fragments was a Gibco-BRL 1 Kb Ladder (taken from the Gibco-BRL 1997-1998 Product Catalogue).



0.5 µg/lane; 1.0% agarose gel stained with ethidium bromide.* *Hind* I fragments of the vector

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